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(54) Title: HETEROLOGOUS G PROTEIN COUPLED RECEPTORS EXPRESSED IN YEAST, THEIR FUSION WITH G PROTEINS AND USE THEREOF IN BIOASSAY

(57) Abstract

The present invention is directed to expression vectors and yeast cells transformed therewith containing a first heterologous nucleotide sequence which codes for a G protein-coupled receptor, for example, the somatostatin receptor, and a second nucleotide sequence which codes for all or a portion of a G protein $\alpha\beta\gamma$ complex. Said heterologous protein is physically expressed in a host cell membrane in proper orientation for both stereoselective binding of ligands, as well as functional interaction with G proteins on the cytoplasmic side of the cell membrane. In some embodiments, a nucleotide sequence encoding a heterologous or chimeric Gα protein is expressed in conjunction with nucleotide sequences from the yeast G protein $\beta\gamma$ subunits. A second aspect of the present invention provides expression vectors and yeast cells transformed therewith encoding chimeric yeast/heterologous G protein coupled receptors. A third aspect of the present invention is directed to methods of assaying compounds using such expression constructs and yeast cell expression systems to determine the effects of ligand binding to the heterologous receptors expressed in the systems.

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Heterologous G protein coupled receptors expressed in yeast, their fusion with G proteins and use thereof in bioassay

Field of Invention

This invention relates to heterologous G protein-coupled receptor expression constructs, yeast cells expressing such receptors, vectors useful for making such cells, and methods of making and using same.

Background of the Invention

The actions of many extracellular signals, for example: neurotransmitters, hormones, odorants and light, are mediated by receptors with seven transmembrane domains (G protein-coupled receptors) and heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins). G proteins are comprised of three subunits: a guanyl-nucleotide binding α subunit; a β subunit; and a γ subunit [for review, see Conklin, B.R and Bourne,H.R. (1993 Cell 73, 631-641]. G proteins cycle between two forms, depending on whether GDP or GTP is bound to the α subunit. When GDP is bound, the G protein exists as a heterotrimer, the G$\alpha\beta\gamma$ complex. When GTP is bound, the α subunit disassociates, leaving a G$\beta\gamma$ complex. Importantly, when a G$\alpha\beta\gamma$ complex operatively associates with an activated G protein coupled receptor in a cell membrane, the rate of exchange of GTP for bound GDP is increased and, hence, the rate of disassociation of the bound Gα subunit from the G$\beta\gamma$ complex increases. The free Gα subunit and G$\beta\gamma$ complex are capable of transmitting a signal to downstream elements of a variety of signal transduction pathways. This fundamental scheme of events forms the basis for a multiplicity of different cell signaling phenomena. For a review, see H.G. Dohlman, J. Thorner, M. Caron, and R. J. Lefkowitz, Ann. Rev. Biochem,

60, 653-688 (1991). G protein-mediated signaling systems are present in organisms as divergent as yeast and man. The yeast *Saccharomyces cerevisiae* is utilized as a model eukaryotic organism. Due to the ease with which one can manipulate the genetic constitution of the yeast *Saccharomyces cerevisiae*, researchers have developed a detailed understanding of many complex biological pathways. It has been demonstrated in numerous systems that the evolutionary conservation of protein structure is such that many heterologous proteins can substitute for their yeast equivalents. For example, mammalian Gα proteins can form heterotrimeric complexes with yeast Gβγ proteins [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590]. The G protein-coupled receptors represent important targets for new therapeutic drugs. Discovery of such drugs will necessarily require screening assays of high specificity and throughput. For example, therapeutic intervention in the somatostatin-growth hormone axis requires new chemical agents that act in a somatostatin receptor subtype-selective manner. The somatostatin receptor (SSTR) is a prototype of the seven transmembrane-domain class of receptors in mammalian cells. The cyclic tetradecapeptide somatostatin, first isolated from hypothalamus and shown to be a potent inhibitor of growth hormone release from the anterior pituitary, has been shown to have broad modulatory effects in CNS and peripheral tissues. In response to binding of somatostatin, SSTR activates a heterotrimeric G protein, which in turn modifies the activity of a variety of effector proteins including but not limited to adenylate cyclases, ion channels, and phospholipases. The effects of somatostatin are transduced through the action of gene products encoded in five distinct receptor subtypes that have recently been cloned [Strnad, J., Eppler, C.M., Corbett, M., and Hadcock, J.R. (1993) BBRC 191, 968-976; Yamada, Y., Post, S.R., Wang, K., Tager, H.S.,

Bell, G.I., and Seino, S. (1992) Proc. Natl. Acad. Sci. USA 89, 251-255; Meyerhof, W., Paust, H.-J., Schonrock, C., and Richter, D. (1991); Kluxen, F.-W., Bruns, C., and Lubbert, H. (1992) Proc. Natl. Acad. Sci. USA 89, 4618-4622; Li, X.-J., Forte, M., North, R.A., Rose, C.A., and Snyder, S. (1992) J. Biol. Chem. 267, 21307-21312; Bruno, J.F., Xu, Y., Song, J., and Berelowitz, M. (1992) Proc. Natl. Acad. Sci. USA 89, 11151-11154; O'Carrol, A.-M., Lolait, S.J., Konig, M., and Mahan, L. (1992) Mol. Pharmocol. 42, 939-946]. Screening assays utilizing yeast strains genetically modified to accommodate functional expression of the G protein-coupled receptors offer significant advantages in research involving ligand binding to the somatostatin receptor, as well as a host of other receptors implicated in various disease states.

Summary of the Invention

A first aspect of the present invention is directed to expression vectors and yeast cells transformed therewith, containing a first heterologous nucleotide sequence which encodes for a G protein-coupled receptor, for example, the somatostatin receptor, and a second nucleotide sequence which encodes for all or a portion of a G protein $\alpha\beta\gamma$ complex. In certain embodiments, all or a portion of a nucleotide sequence encoding for a heterologous G protein α subunit is fused to a nucleotide sequence from the yeast G protein α subunit. In certain preferred embodiments, the expression vectors and transformed cells contain a third heterologous nucleotide sequence comprising a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. The vectors and cells may further contain several mutations. These include 1) a mutation of the *yeast SCG1/GPA1* gene, which inactivates the

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yeast Gα protein, facilitating interaction of the heterologous receptor with the G protein; 2) a mutation of a yeast gene to inactivate its function and enable the yeast cell to continue growing in spite of activation of the pheromone response signal transduction pathway, preferred embodiments being mutations of the *FAR1* and/or *FUS3* genes; and, 3) a mutation of a yeast gene, the effect of the which is to greatly increase the sensitivity of the response of the cell to receptor-dependent activation of the pheromone response signal transduction pathway, preferred genes in this regard being the *SST2, STE50, SGV1, STE2, STE3, PIK1, AFRI, MSG5,* and *SIG1* genes.

A second aspect of the present invention is a chimeric expression construct and yeast cells transformed therewith comprising a first nucleotide sequence encoding for a yeast G protein coupled receptor in operative association with a heterologous nucleotide sequence which encodes for a heterologous G protein coupled receptor. The constructs and cells may contain a second heterologous nucleotide sequence comprising a pheromone-responsive promotor and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. The constructs and cells may further contain several mutations. These include 1) a mutation of a yeast gene to inactivate its function and enable the yeast cell to continue growing in spite of activation of the pheromone response signal transduction pathway, preferred embodiments being mutations of the *FAR1* and/or *FUS3* genes; and, 2) a mutation of a yeast gene, the effect of the which is to greatly increase the sensitivity of the response of the cell to receptor-dependent activation of the pheromone response signal transduction pathway, preferred genes in this regard being the *SST2, STE50, SGV1, STE2, STE3, PIK1, AFRI, MSG5,* and *SIG1* genes. A productive signal is detected in a bioassay through coupling of the heterologous receptor to a

yeast protein.

A third aspect of the present invention is a method of assaying compounds to determine effects of ligand binding to the heterologous receptors by measuring effects on cell growth. In certain preferred embodiments, yeast cells of the kind described above are cultured in appropriate growth medium to cause expression of heterologous proteins, embedded in agar growth medium, and exposed to compounds applied to the surface of the agar plates. Effects on the growth of embedded cells are expected around compounds that activate the heterologous receptor. Increased growth may be observed with compounds that act as agonists, while decreased growth may be observed with those that act as antagonists.

Brief Description of the Drawings

FIGURE 1. Strains containing the indicated Gα expression plasmids are treated with mating pheromone (α factor). A measure of resulting signal transduction is provided by a reporter plasmid carrying *FUS1-lacZ*. Data are represented as a percent of β-galactosidase activity measured in a strain expressing solely the wild type Gα protein.

FIGURE 2. Strains containing the indicated CUP1p-Gα expression plasmid and grown in medium containing the indicated concentration of copper are treated with mating pheromone (α factor). A measure of resulting signal transduction is provided by a reporter plasmid carrying FUS1-lacZ. Data are represented as a percent of β-galactosidase activity measured in a strain expressing no exogenous Gα protein.

FIGURE 3. Saturation binding of ^{3}H-spiperone to yeast membrane fractions prepared from a strain (CY382)

expressing the 5HT1a serotonin receptor. Bmax = 3.2 pmol/mg protein; Kd - 115 nM.

FIGURE 4. Amino-terminal chimeric Ste2/5HT1a receptors. The CHI11 receptor contains the first 14 amino acids of the yeast Ste2 protein. The CHI17 receptor has a replacement of the amino-terminus of the 5HT1a receptor through the first two transmembrane domains with the corresponding region of the Ste2 receptor. The CHI18 receptor has the same Ste2 sequences fused directly to the amino-terminus of the 5HT1a receptor to create a receptor predicted to span the cellular membrane nine times. Bmax values were determined by measuring maximal binding of the radiolabeled ligand 3H-spiperone. Values are given as pmol radioligand bound per mg total protein.

FIGURE 5. Competition binding analysis of the agonists isoproterenol or epinephrine against ^{125}I-cyanopindolol with crude membrane extracts prepared from a wild-type yeast strain expressing the β_2-adrenergic receptor. Data are presented as percent maximal radioligand binding. IC_{50} values = 10 nM, isoproterenol; 200 nM, epinephrine.

FIGURE 6. Competition binding analysis of the agonists isoproterenol or epinephrine against ^{125}I-cyanopindolol with extracts prepared from a yeast strain coexpressing the β_2-adrenergic receptor and mammalian G_{as}. Data are presented as percent maximal radioligand binding. IC_{50} values = 10nM,isoproterenol; 60 nM, epinephrine.

FIGURE 7. Saturation binding of [^{125}I]tyr^{11}S-14 to membranes of yeast cells coexpressing the SSTR2 subtype and Scg1/G_{ia2}. Membranes from yeast cells expressing the SST2 subtype were prepared as described in *Experimental Procedures*. Saturation binding was performed with 20-1600 pM [^{125}I]tyr^{11}S-14. Non-specific binding for each point as cpm bound in the presence of 1 μM cold S-14 ranged from 10 to 40%. Displayed is a representative experiment performed

in duplicate.

FIGURE 8. Immunoblot showing somatostatin receptor expression. Membrane fractions are isolated from the indicated yeast strains. Aliquots of 5 to 30 µg of protein are examined by polyacrylamide gel electrophoresis/Western blot analysis. Molecular weight markers are indicated in kilodaltons. Arrows mark somatostatin receptor protein bands. Several species of this receptor are observed; doublet and triplet bands in addition to the 43 kd single receptor. Lanes, 1+2, CY602 (see Table 1); 3+4. CY603; 5+6, CY624; 7, congenic strain expressing no receptor.

FIGURE 9. Immunoblot showing muscarinic acetylcholine receptor (mAchR) expression. Membrane fractions are isolated from the indicated yeast strains. Aliquots of 30 µg of protein are examined by polyacrylamide gel electrophoresis/Western blot analysis as described in the text. Molecular weight markers are indicated in kilodaltons. Arrows mark mAchR protein bands.

FIGURE 10. Immunoblot showing α2-AR expression. Membrane fractions are isolated form the indicated yeast strains. Aliquots of 30 µg of protein are examined by polyacrylamide gel electrophoresis/ western blot analysis. Molecular weight markers are indicated in kilodaltons. Arrows mark α2-AR protein bands.

FIGURE 11. Somatostatin receptor expression plasmid, pJH1.

FIGURE 12. G protein expression plasmid, pLP82.

FIGURE 13 (A&B). Dose dependent growth response of yeast cells to somatostatin. Cultures of yeast strain LY268 are embedded in agar (top plate) or spread evenly on the surface of agar plates (bottom plate) and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 14(A,B,C,&D). Growth response of yeast strains exposed to somatostatin is dependent on amount of chimeric G protein expressed. Cultures of yeast strains described in the text are embedded in agar and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE15(A,B,C,&D) Growth response of yeast strains exposed to somatostatin is dependent on amount of yeast G protein expressed. Cultures of yeast strains described in the text are embedded in agar and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 16 (A&B). Yeast cells bearing a mutation in the *sst2* gene exhibit elevated resistance to AT when exposed to mating pheromone. Cultures of yeast strains are embedded in agar and exposed to the indicated amounts of α mating factor spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 17 (A,B,C,&D). A mutation in the *sst2* gene enhances the growth of the yeast cells exposed to somatostatin. Cultures of yeast strains described in the text are embedded in agar and exposed to the indicated amounts of designated compounds spotted on paper disks placed on top of the agar. Plates are incubated at 30°C.

FIGURE 18. Ligand-binding to the rat CCK_B receptor expressed in yeast. Crude membrane fractions from overnight liquid cultures of LY631 cells were prepared, and agonist saturation binding assays conducted as described in Methods and Materials. Saturation binding was performed with 4-60 nM[^{3}H] CCK-8 (25 µg protein/tube). Non-specific binding for each point as cpm bound in the presence of 1 µM cold CCK-8 ranged from 20 to 60%. Displayed is a representative experiment performed in duplicate.

FIGURE 19 (A&B). Growth of yeast in response to CCK_B

receptor agonists. Yeast strains that functionally express the rat CCK_B receptor (LY628, LLY631) were cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing 10 µl amounts of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(A)** CCK-8, **(B)** CCK-4.

FIGURE 20. A_{2a}-adenosine receptor saturation binding assay. Radioligand binding assays were performed in 96-well microliter plates using binding buffers (50nM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.25% BSA) containing protease inhibitors (5µg/ml leupeptin, 5 µg/ml aprotinin, 100 µg/ml bacitracin, and 100 µg/ml benzamidine). All components were diluted in binding buffer containing protease inhibitors and added to the microliter plate wells in the following order: binding buffer, cold competitor (NECA, 1 µM final concentration), [^{3}H]NECA (1-50nM). Binding reactions were initiated by adding 82 µg of membrane protein in a 170 µl volume. Final reaction volume was 200 µl/well. All incubations were carried out at room temperature for 2 hours. Free radioligand was separated from bound ligand by rapid filtration through a glass fiber filter using an Inotech cell harvester. The filter disks were then washed several times with cold (4°C) binding buffer lacking BSA prior to counting.

FIGURE 21. Growth of yeast in response to A_{2A}-adenosine receptor agonists. LY595 cells cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing the 10 µg of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(A, B)** CGS-21680, **(B)** NECA **(C)** DPMA.

FIGURE 22. Growth of yeast cells containing SSTR5 in response to somatostatin receptor agonists. LY620 cells cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(A)** 60 nmol S-14, (B) 30 nmol S-28.

Figure 23. Growth of yeast cells containing porcine SSTR2 in response to somatostatin receptor agonists. LY474 (two independent isolates: 21,22) were cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(1)** 600 pmol, **(2)** 60 pmol.

FIGURE 24. Deletion of *MSG5* increases the sensitivity of the yeast bioassay. Cultures of yeast strains were induced to express the SSTR2 as described in Materials and Methods and were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of S-14. The plates were then incubated at 30°C for 3 days. **(A)** MPY459 *sst2ΔADE2 msg5ΔLEU2,* **(B)** MPY458 *SST2 msg5ΔLEU2,* **(C)** LY288 *SST2 MSG5,* **(D)** LY268 *sst2ΔADE2 MSG5.*

FIGURE 25 (A&B). Growth of yeast in response to GRF receptor agonists. CY990 cells cultured as described in Materials and Methods were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). Sterile filter disks were placed on the surface of the solidified agar and

saturated with 10 µl of sterile water containing 20 nmol of the indicated compounds. The plates were then incubated at 30°C for 3 days. **(A)** hGRF(1-29)-NH_2, **(B)** hGRF (1-29), (D-arg^2)-hGRF(1-29).

FIGURE 26 (A&B). Effect of *STE50* overexpression on SSTR2 bioassay. Assay medium and yeast strains were prepared as described in Materials and Methods. Plate A contains the *STE50* overexpression strain CY560; plate B contains the control strain CY562. Filter discs saturated with solutions of the following peptides were applied to each plate: 1 mM yeast α pheromone (lefthand center), 1 µg/ml somatostatin-14 (righthand top), 100 µg/ml somatostatin-14 (righthand bottom). Plates were incubated at 30° C for 3 days.

FIGURE 27 (A&B). Bioassay of compounds with somatostatin receptor and/or antagonist properties. LY364 cells were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cells/ml). For assay of antagonists, somatostatin (20 nM S-14) was added to the molten agar prior to pouring. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing test compounds. The plates were then incubated at 30°C for 3 days **(Left)** Assay for somatostatin agonists. Somatostatin (S-14) was applied to positions on the bottom row, left side (6 nmol, 600 pmol, 60 pmol, 600 pmol), **(Right)** Assay for somatostatin antagonists. Somatostatin (S-14) was applied to positions on the bottom row, left side (6 nmol, 600 pmol, 60 pmol, 600 pmol).

FIGURE 28 (A&B) Fusion of STE2 sequences to the amino terminal of SSTR2 reduces signaling effeciency in response to somatostatin. LY268 and LY322 cells were plated in SC Galactose (2 %)-ura, trp, his agar medium (2 x 10^4 cell/ml). Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of somatostatin (S-

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14). The plates were then incubated at 30°C for 3 days. (A) LY268. S-14 was applied to filter disks clockwise from the top: carrier, 60 nmol, 6 nmol, 600 pmol, 60 pmol, 6 pmol. (B) LY322. S-14 was applied to filter disks clockwise from the top: 0.6 pmol, carrier, 60 nmol, 6 nmol, 600 pmol, 60 pmol, 6 pmol.

Detailed Description of the Invention

Nucleotide bases are abbreviated herein as follows:

A-Adenine	G-Guanine
C-Cytosine	T-Thymine

U-Uracil (sometimes herein abbreviated as "ura")

Amino acid residues are abbreviated herein to either three letters or a single letter as follows:

Ala;A-Alanine	Leu;L-Leucine
Arg;R-Arginine	Lys;K-Lysine
Asn;N-Asparagine	Met;M-Methionine
Asp;D-Aspartic acid	Phe;F-Phenylalanine
Cys;C-Cysteine	Pro;P-Proline
Gln;Q-Glutamine	Ser;S-Serine
Glu;E-Glutamic acid	Thr;T-Threonine
Gly;G-Glycine	Trp;W-Tryptophan
His;H-Histidine	Tyr;Y-Tyrosine
Ile;I-Isoleucine	Val;V-Valine

The terms "DNA" and "nucleotide sequence" are used interchangably and are meant to include all forms of linear polymers comprising nucleotide bases, without limitation, including RNA when appropriate.

The term "mammalian" as used herein refers to any mammalian species (e.g. human, mouse, rat, and monkey).

The term "heterologous" is used herein with

respect to yeast, and hence refers to DNA sequences, proteins, and other materials originating from organisms other than yeast (e.g., mammalian, avian, amphibian, insect, plant), or combinations thereof not naturally found in yeast.

The term "upstream" and "downstream" are used herein to refer to the direction of transcription and translation, with a sequence being transcribed or translated prior to another sequence being referred to as "upstream" of the latter.

Any G protein-coupled receptor, or portions thereof, as well as the nucleotide sequences encoding same, may be employed in practicing the present invention. Examples of such receptors include, but are not limited to, adenosine receptors, somatostatin receptors, dopamine receptors, cholecystokinin receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors, growth hormone releasing factor, glucagon, and serotonin receptors. The term receptor as used herein is intended to encompass subtypes of the named receptors, and mutants and homologs hereof, along with the nucleotide sequences encoding same. One skilled in the art will also understand that in some instances, it may not be necessary that the entire receptor be expressed to achieve the purposes desired. Accordingly, the term receptor is meant to include truncated and other variant forms of a given receptor, without limitation.

Any DNA sequence which codes for a Gα subunit (Gα) may be used to practice the present invention. Examples of Gα subunits include, but are not limited to Gs subunits, Gi subunits, Go subunits, Gz subunits, Gq, G11, G16 and transducing subunits. G proteins and subunits useful for practicing the present invention include subtypes, and mutants and homologs thereof, along with the DNA sequences encoding same.

One skilled in the art will understand from the teachings as presented herein that the G proteins useful in the constructs and yeast cells of the present invention may comprise neterologous Gα subunits, yeast Gα subunits, or chimeric yeast/neterologous versions. One can easily determine which configuration is best suited for adequate coupling to a particular heterologous receptor by simply constructing vectors as taught herein and measuring the signaling of ligand binding in response to a given assay. In certain preferred embodiments, $G\alpha_{i2}$ is the Gα subunit of choice, particularly when the heterologous G coupled protein is all or a portion of a somatostatin receptor. It is particularly preferred in this instance that the $G\alpha_{i2}$ subunit be coupled to a yeast Gβγ complex. Certain chimeric constructs may also provide enhanced signal transduction with regard to particular heterologous receptors. Particularly preferred is a chimeric construct formed from fusion of the amino terminal domain of yeast *GPA1/SCG1* with the carboxy terminal domain of a heterologous $G\alpha_i$, $G\alpha_s$, and especially $G\alpha_{i2}$.

Any DNA sequence which codes for a Gβγ subunit (Gβγ) may be used to practice the present invention. G proteins and subunits useful for practicing the present invention include subtypes, and mutants and homologs thereof, along with the DNA sequences encoding same. The host cells may express endogenous Gβγ, or may optionally be engineered to express heterologous Gβγ (e.g., mammalian) in the same manner as they would be engineered to express heterologous Gα.

Heterologous DNA sequences are expressed in a host by means of an expression "construct" or "vector". An expression vector is a replicable DNA construct in which a DNA sequence encoding the heterologous DNA sequence is operably linked to suitable control sequences capable of affecting the expression of a protein or protein subunit

coded for by the heterologous DNA sequence in the intended host. Generally, eukaryotic control sequences include a transcriptional promoter, however, it may be appropriate that a sequence encoding suitable mRNA ribosomal binding sites be provided, and (optionally) sequences which control the termination of transcription. Vectors useful for practicing the present invention include plasmids, viruses (including bacteriophage), and integratable DNA fragments (i.e., fragments integratable into the host genome by genetic recombination). The vector may replicate and function independently of the host genome, as in the case of a plasmid, or may integrate into the genome itself, as in the case of an integratable DNA fragment. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. For example, a promoter operable in a host cell is one which binds the RNA polymerase of that cell, and a ribosomal binding site operable in a host cell is one which binds the endogenous ribosomes of that cell.

DNA regions are operably associated when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, contiguous and in reading phase.

Transformed host cells of the present invention are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques and express the protein or protein subunit coded for by the heterologous DNA sequences. A variety of yeast cultures, and suitable expression vectors for transforming yeast cells, are known. See e.g., U.S. Patent No. 4,745,057; U.S. Patent No. 4,797,359; U.S. Patent No. 4,615,974; U.S. Patent

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No. 4,880,734; U.S. Patent No. 4,711,844; and U.S. Patent No. 4,865,989. *Saccharomyces cerevisiae* is the most commonly used among the yeasts, although a number of other yeast species are commonly available. See. e.g., U.S. Patent No. 4,806,472 (*Kluveromyces lactis* and expression vectors therefore); 4,855,231 (*Pichia pastoris* and expression vectors therefore). Yeast vectors may contain an origin of replication from the endogenous 2 micron yeast plasmid or an autonomously replicating sequence (ARS) which confers on the plasmid the ability to replicate at high copy number in the yeast cell, centromeric (CEN) sequences which limit the ability of the plasmid to replicate at only low copy number in the yeast cell, a promoter, DNA encoding the heterologous DNA sequences, sequences for polyadenylation and transcription termination, and a selectable marker gene. Exemplary plasmids and detailed of materials and methods for making and using same are provided in the Examples section.

Any promoter capable of functioning in yeast systems may be selected for use in the constructs and cells of the present invention. Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (*PGK*) [Hitzeman et al., (1980) J.Biol. Chem. 255, 2073] or other glycolytic enzymes [(Hess et al., (1968) J. Adv. Enzyme Reg. 7, 149]; and Holland et al., (1978) Biochemistry 17, 4900], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate, decarboxylase,phosphofructokinase, glucose-6-phosphate isomerase, 3- phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase, 1,2,-isocytochrome C, acid phosphates,

degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization, such as the galactose inducible promoter, *GAL1*. Particularly preferred for use herein are the *PGK*, *GAL1*, and alcohol dehydrogenase (ADH) promoters. Finally, in constructing suitable expression plasmids, the termination sequences associated with these genes may also be ligated into the expression vector 3' of the heterologous coding sequences to provide polyadenylation and termination of the mRNA. In preparing the preferred expression vectors of the present invention, translational initiation sites are chosen to confer the most efficient expression of a given nucleic acid sequence in the yeast cell [see Cigan, M. and T.F. Donahue 1987, GENE, Volume 59, pp. 1-18, for a description of suitable translational initiation sites].

A particularly preferred nucleotide expression vector useful for carrying out the present invention comprises such an aforementioned promoter sequence, positioned upstream to the translational initiation site of the heterologous nucleotide sequence encoding for the heterologous G protein coupled receptor it is desired to express, and in correct reading frame therewith. Particularly preferred promoters in this regard are the *GAL1*, *PGK*, and *ADH* promoters. Positioning of the aforementioned promotor upstream to the chosen translational initiation site may enhance expression of a heterologous protein. In these preferred embodiments, no yeast G protein coupled receptor segment is fused to the heterologous G protein coupled receptor segment. The present inventors have discovered that such hybrid receptors are not critical to achieve receptor expression in yeast. This is contrary to the art accepted teaching in this regard [see King, et al. cited *infra*].

In certain other embodiments however, at least a

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fragment of the 5'-untranslated region of a yeast gene is positioned upstream from the heterologous G protein coupled segment and operatively associated therewith. To that end, the present invention also provides constructs having suitable promoters and translational initiation sites as described above, but these constructs include a yeast segment comprising at least a fragment of the extreme amino-terminal coding nucleotide sequence of a yeast G protein-coupled receptor and a second segment downstream from said first segment and in correct reading frame therewith, the second segment comprising a nucleotide sequence encoding a heterologous G protein-coupled receptor. The yeast segment in this regard may be provided to actually act as a reporter sequence, rather than to serve to enhance effective expression of the heterologous G protein in the yeast system. Thus, certain embodiments comprise a gene sequence encoding a yeast segment of a yeast G protein-coupled receptor, that acts as a reporter segment, in that it encodes a peptide that may be detected through conventional means, such as antibody binding, and the like. Preferred in this regard is all or a portion of a yeast pheromone receptor fused to a heterologous G protein coupled receptor, which may be used primarily as an "epitope tag" for the highly specific detection of expression of the desired heterologous receptor using antibodies directed specifically to the epitope sequence expressed. In constructing such a vector, the yeast segment may be positioned upstream to the heterologous protein, or alternatively, a fragment of the extreme amino-terminal coding sequence of the heterologous G protein-coupled receptor may be deleted, and the yeast segment fused directly thereto. In some cases, one or more of the amino terminal transmembrane domains or intracellular domains of the heterologous protein are deleted. Alternatively, the yeast segment may be added directly to the amino terminus of the heterologous receptor, thereby

elongating the overall chimeric receptor construct.

The first and second segments are operatively associated with a promoter, such as the *GAL1* promoter, which is operative in a yeast cell. Coding sequences for yeast G protein-coupled receptors which may be used in constructing such vectors are exemplified by the gene sequences encoding yeast pheromone receptors (e.g., the *STE2* gene, which encodes the α-factor receptor, and the *STE3* gene, which encodes the **a**-factor receptor).

Certain preferred chimeric receptors provided herein comprise a yeast *Ste2* protein segment fused directly to all or a portion of a heterologous G protein receptor, and preferably, the 5HT1a receptor, muscarinic receptor, α-adrenergic receptor, or a somatostatin receptor.

Any of a variety of means for detecting the effects of ligand binding can be utilized. For example, measurement of the disassociation of Gα from Gβγ can be made through conventional biochemical techniques. However, it should be noted that the binding of ligand to a receptor may either trigger or block a detectable biological response, which may also lend itself to measurement. One such biological response is the ability of yeast cells to mate. Use of the pheromone induced mating signal transduction pathway is a preferred method of detecting the effects of ligand binding in the assay systems herein presented, the basic premise of which is discussed in more detail, as follows.

G protein-coupled pheromone receptors in yeast control a developmental program that culminates in mating (fusion) of a and α haploid cell types to form the a/α diploid (for a review, see G.F. Sprague, Jr. and J. W. Thorner, in the Molecular Biology and Cellular Biology of the Yeast Saccharomyces: volume II, Gene Expression). The process of mating is initiated by extracellular peptides, the mating pheromones. Cells of the **a** mating type secrete

α-factor, which elicits a response in α-cells; cells of the α-mating type secrete **a**-factor which acts only on **a** cells. Haploid cells respond to the presence of the peptide mating pheromones through the action of endogenous G protein-coupled pheromone receptors (*STE2*: the α-factor receptor, expressed only in α cells and *STE3*: the **a**-factor receptor expressed only in **a**-cells). Both receptors interact with the same heterotrimeric G proteins and a signal transduction cascade that is common to both haploid cell types. Upon pheromone-binding to receptor, the receptor presumably undergoes a conformational change leading to activation of the G protein. The α-subunit, *SCG1/GPA1*, exerts a negative effect on the pheromone response pathway, which is relieved by receptor-dependent activation. The complex of βγ subunits (*STE4, STE18*) is thought to transmit the positive signal to an effector, possibly *STE20*, a putative protein kinase [Leberer, E., Dignard, D., Harcus, D., Thomas, D.Y., Whiteway, M. (1992) EMBO J. 11, 4815-4824]. The effector in turn activates downstream elements of the signal transduction pathway which include *STE5*, and a presumptive protein kinase cascade composed of the products of the *STE11*, *STE7*, *FUS3* and *KSS1* genes, eventually resulting in cell cycle arrest and transcription induction. The primary interface between elements of the pheromone response pathway and cell cycle regulatory machinery is the *FAR1* gene product. Certain recessive alleles of *FAR1* and *FUS3* fail to undergo cell cycle arrest in response to pheromone, while permitting pheromone dependent transcription to occur. Pheromone-dependent transcription is mediated through the action of the sequence-specific DNA-binding protein *STE12*. Activation of *STE12* results in transcription of genes possessing a cis-acting DNA sequence, the pheromone response element. These pheromone responsive genes encode products that are required for pheromone synthesis (*MFa1*, *MFa2*, *MFA1*, *MFA2*, *STE6*, *STE13*) and the response to pheromone (*STE2*,

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STE3, SCG1/GPA1, FUS3), facilitate or participate in cell association and fusion (*FUS1*), cell cycle arrest (*FAR1*), and the morphological events required for mating. In the event that the mating process is not consummated, yeast cells become adapted to the presence of pheromone and resume mitotic growth. Thus, in certain preferred embodiments, the *FUS3* or *FAR1* gene is mutated or deleted altogether, thereby disconnecting the cell cycle arrest pathway from the signal transduction pathway, and allowing continued growth of the cells in response to mating pheromone binding to the heterologous receptor. Since *FAR1* is a primary factor in the cell cycle regulatory pathway, its deletion or mutation is preferred in the expression constructs of the present invention. Yeast cells transformed with such constructs yield superior yeast strains for ligand-binding assays.

The mating signal transduction pathway is known to become desensitized by several mechanisms including pheromone degradation and modification of the function of the receptor, G proteins and/or downstream elements of the pheromone signal transduction by the products of the *SST2, STE50, AFR1* [Konopka, J.B. (1993) Mol. Cell. Biol. 13, 6876-6888] and *SGV1, MSG5,* and *SIG1* genes. Selected mutations in these genes can lead to hypersensitivity to pheromone and an inability to adapt to the presence of pheromone. For example, introduction of mutations that interfere with function into strains expressing heterologous G protein-coupled receptors constitutes a significant improvement on wild type strains and enables the development of extremely sensitive bioassays for compounds that interact with the receptors. Other mutations e.g. *STE50, sgv1, ste2, ste3, pik1, msg5, sig1, and afr1,* have the similar effect of increasing the sensitivity of the bioassay. One skilled in the art will understand that increased sensitivity of the assay systems is attained through deletion of one or more of these aforementioned genes, introduction of mutations that

down-regulate their expression, or in certain instances, effecting their overexpression. For example, in the *STE50* construct, overexpression of the gene is desired, not deletion of the gene.

Introduction of a constellation of mutations in the mating signal transduction pathway results in a yeast cell well suited to expression of heterologous G protein-coupled receptors, which are able to functionally respond to their cognate ligands, while providing a biological response that signals the binding of the receptor to the ligand.

In conjunction with one or more of the above-referenced mutations, a particularly convenient method for detecting ligand-binding to heterologous receptor expressed in yeast cells is to utilize a conventional genetic indicator system. Thus, in certain preferred embodiments, the cells are provided with an additional heterologous nucleotide sequence, comprising a pheromone-responsive promoter and an indicator gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith. With such a sequence in place, the detecting step can be carried out by monitoring the expression of the indicator gene in the cell. Any of a variety of pheromone responsive promoters could be used, examples being promoters driving any of the aforementioned pheromone responsive genes (*e.g. mFa1, mFa2, MFA1, MFA2, STE6, STE13)*, the BAR1 gene promoter, and the FUS1 gene promoter. Likewise, any of a broad variety of indicator genes could be used, with examples including the *HIS3, G418r, URA3, LYS2, CAN1, CYH2,* and *LacZ* genes. A particularly preferred reporter gene construct is utilized by fusing transcription control elements of a *FUS1* gene to *HIS3* protein coding sequences, and replacing the original *FUS1* gene with this reporter construct. Expression of the *HIS3* gene product is thereby placed under the control of the pheromone signal transduction pathway. Yeast strains (*his3*) bearing this

construct are able to grow poorly on supplemented minimal medium lacking histidine, and are sensitive to an inhibitor of the *HIS3* gene product. In other preferred embodiments, plasmids carry a *FUS1-lacZ* gene fusion. Expression of the *FUS1* gene is stimulated in response to receptor activation by binding of pheromone. Therefore, signal transduction can be quantitated by measuring β-galactosidase activity generated from the *FUS1-lacZ* reporter gene.

Other useful reporter gene constructs, still under the control of elements of the pheromone signal transduction pathway, but alternative to the above-discussed reporter systems, may involve signals transduced through other heterologous effector proteins that are coexpressed. For example, 1) ligand-dependent stimulation of a heterologous adenylylcyclase may permit a yeast strain lacking its own adenylylcyclase due to mutation in the cdc35 gene to survive, 2)ligand-dependent stimulation of a heterologous G protein-coupled potassium channel may permit a yeast strain unable to grow in medium containing low potassium concentration [(*trk1, trk2*), for example, see Anderson, J.A. et al (1992)(Proc. Natl. Adad. Sci. USA 89, 3736-3740] to survive, or 3) ligand-dependent stimulation of a heterologous phospholipase C (especially PLC-β) may permit a yeast strain lacking its own PLC [(plc), for example, see Payne, W.E. and Fitzgerald-Hayes, M. (1993) Mol. Cell Biol. 13,4351-4363] to survive.

Any DNA sequence which codes for an adenylylcyclase may be used to practice the present invention. Examples of adenylylcyclase include the product of the D. melanogaster Rutabaga gene and the mammalian subunit types I-VIII [for review see, Tang, W.-J. and Gilman, A.G. (1992) Cell 70, 869-872], and mutants and homologs thereof, along with the DNA sequences encoding same, which are useful for practicing the present invention.

Any DNA sequence which codes for a G protein-gated

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potassium channel may be used to practice the present invention. Examples of G protein-coupled potassium channel include GIRK1 [Kubo, Y. Reuveny, E., Slesinger, P.A., Jan, Y.N., and Jan, L.Y. (1992) Nature 365, 802-806], subunits useful for practicing the present invention, and mutants and homologs thereof, along with the DNA sequences encoding same.

Any DNA sequence which codes for a phospholipase protein may be used to practice the present invention. Examples of phospholipase (PLC) proteins include the D. melanogaster norpA gene product and the PLC-β proteins [for review, see Rhee, S.G., and Choi, K.D. (1992) J. Biol. Chem. 267, 12392-12396], subunits useful for practicing the present invention, and mutants and homologs thereof, along with the DNA sequences encoding same.

A particularly preferred yeast expression system is described herein, having yeast cells bearing SSTR and chimeric G-protein, and dependent upon the presence of somatostatin for continued growth. As noted above, transformed host cells of the present invention express the proteins or protein subunits coded for by the heterologous DNA sequences. When expressed, the G protein-coupled receptor is located in the host cell membrane (i.e., physically positioned therein in proper orientation for both the stereoselective binding of ligands and for functional interaction with G proteins on the cytoplasmic side of the cell membrane). Implementation of the sensitive and specific yeast expression system described herein will facilitate description of structural and functional aspects of receptor-ligand and receptor-G protein interactions. Powerful genetic selection schemes, made possible by modification of elements of the mating signal transduction pathway, may be employed to identify aspects of the receptor that have effects on agonist selectivity, ligand stereo selectivity, and determinants of agonist/antagonist binding.

The role of proteins that modify the response of receptors and G proteins to ligand may be worked out in detail with the assistance of this powerful genetic system. Importantly, the system provides a generalized approach to the study of the functioning and components of the G protein-coupled signal transduction system, as well as a generalized approach to screening assays utilizing the G protein coupled signal transduction system. The present invention provides expression constructs and assay systems adapted to receive any of a variety of heterologous G protein coupled receptors, in the form of "expression cassettes". The heterologous G protein-coupled receptor it is desired to study is simply inserted into the vectors herein provided, and expressed in yeast cells. Ligands that may bind to the expressed receptor are allowed to come into contact with the cells in any conventional assay manner, and the effects of the interaction are easily monitored. The systems presented herein thus provide tremendous utility in the identification of ligands for orphan G protein-coupled receptors and for discovering novel therapeutically useful ligands for receptors of medical, veterinary, and agricultural importance.

The following Examples are provided to further illustrate various aspects of the present invention. They are not to be construed as limiting the invention.

EXAMPLE 1

Functional expression of mammalian G α proteins in *Saccharomyces cerevisiae*.

A sensitive bioassay is utilized to measure interference of yeast Gα and Gβγ interactions by expression of heterologous Gα proteins. Mammalian Gα genes are

expressed from 2μ or centromere-bearing plasmids under the control of the constitutive *PGK* or the inducible *CUP1* promoter. The data demonstrates that the rat $G\alpha_s$, $G\alpha_{i2}$, and chimeric yeast/mammalian Gα can effectively interact with yeast Gβγ.

Media and Strains. Growth of bacterial strains and plasmid manipulations are performed by standard methods (Maniatis T., Molecular Cloning, (Cold Spring Harbor Laboratory Press, 1982). Growth and transformation of yeast strains are performed as described in Rose et al. (Rose M.D., Methods in yeast genetics, Cold Spring Harbor Laboratory Press, 1990). The yeast strains used in these studies (CY414, *MATa ura3-52 trp1 leu2 his3 pep4::HIS3*) originate from strains described by E.Jones (Jones, E.W., Ann,. Rev. Genet 18:233, 1984). CY414 is sequentially transformed with the *FUS1-lacZ* fusion plasmid pSB234 (Trueheart J., et al Mol. Cell. Biol. 7(7): 2316-2328, 1987) and Gα expression plasmids.

Construction of G α expression plasmids. Rat cDNA clones for $G\alpha_s$ and $G\alpha_{i2}$ and for fusions with the yeast *SCG1* gene are described elsewhere [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590]. To express these genes from low-copy-number plasmids, XhoI-SalI fragments containing each expression cassette (including the *PGK* promoter and terminator sequences) are isolated and cloned into the CEN plasmid pRS414 digested with XhoI. For inducible expression, the DNA segment containing *PGK* promoter sequences are replaced with upstream activating sequences form the *CUP1* gene.

β-galactosidase assays. Cultures are diluted to $5x10^7$ cells/ml and aliquotted into separate tubes. Pheromone is added to a final concentration of 10^{-9}M to one sample. Cultures are then incubated for 4 hrs at 30°C. Subsequent measurement of β-galactosidase activity is conducted as described elsewhere (Rose M.D., Cold Spring

Harbor Laboratory Press, 1990).

High and low-copy-number plasmids carrying the yeast *SCG1* or mammalian $G\alpha_s$ or $G\alpha_i$ or chimeric yeast/mammalian Gα genes expressed from the yeast *PGK* promoter are transformed into a wild-type yeast strain also containing a plasmid carrying a *FUS1-lacZ* gene fusion. Expression of the *FUS1* gene is stimulated in response to receptor activation by binding of pheromone. Therefore, signal transduction can be quantitated by measuring β-galactosidase activity generated from the *FUS1-lacZ* reporter gene. Interference of normal signal transduction by expression of a heterologous Gα protein is observed as a decrease in β-galactosidase activity.

Strains expressing introduced Gα genes are assayed for pheromone-induced gene activation. Data are represented as percent of wild-type response in Figure 1. Expression from all Gα plasmids reduced *FUS1-lacZ* expression levels demonstrating that the Gα proteins functionally coupled to yeast Gβγ. A dose dependence was observed for Scg1, Gαs and $G\alpha_{12}$. Expression from high-copy-number plasmids greatly reduces signaling suggesting that a large excess of heterologous Gα protein is present. The Scg-Gαi2 chimeric protein reduces signaling to near the unstimulated background levels even from the low-copy-number plasmid. Other CEN expression plasmids reduce signaling to 53 to 84% of wild-type levels.

To achieve more precise control of Gα expression and reduce expression to a level sufficiently low that minimal effects on pheromone induced signaling will occur, Gα genes (except Gαs) are placed under the control of the inducible *CUP1* promoter and transformed into yeast on low-copy-number plasmids. The level of signaling repression mediated by these plasmids is dependent on the concentration of Cu++4 added to the medium (Figure 2). However, basal expression (no Cu++4 added) was equivalent to levels

observed from the *PGK* promoter (Figure 1). As in the previous experiment, the *SCG*-Gαi2 chimeric protein reduces signaling to almost background levels.

The data presented in Figures 1 and 2 indicates that all Gα expression plasmids examined produce functional Gα proteins in that all inhibit the signal transduction pathway. Using a constitutive promoter (*PGK*), most Gα genes exhibit a dose-dependent effect with high-copy-number 2 micron plasmids drastically reducing signaling (Figure 1). Lower expression from CEN plasmids reduce signaling levels as little as 16% (See G_i, Figure 1). Expression of the Gα genes from the CUP1 promoter shows expected dose/response effects with reduced signaling correlated to increased Cu++ concentrations (Figure 2).

EXAMPLE 2

Pharmacological evaluation of heterologous G protein-coupled receptors expressed in *Saccharomyces cerevisiae*

Yeast strains. Growth and transformation of yeast strains are performed as described (Rore, M.D., Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, 1990). The yeast strains used in these studies (CY414; *MATa ura3-52 trp1 leu2 his3 pep4ΔHIS3*) originate from strains described by E. Jones (Jones, E. W., Ann. Rev. Genet, 18:233, 1984).

Nucleic acid manipulation. Growth of bacterial strains and plasmid manipulations are performed by standard methods [Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning, 2nd ed. (Cold Spring Harbor Laboratory Press, 1989)]. DNA sequencing is performed by high temperature cycle sequencing (Applied Biosystems).

Protein analysis. Receptor expression strains are

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grown in synthetic complete medium lacking specific nutrients to select for plasmid retention and containing 3% galactose to induce receptor gene expression. Cells are pelleted and washed in lysis buffer (10 mM sodium bicarbonate, pH 7.2, 1 mM EGTA, 1 mM EDTA) then resuspended in lysis buffer plus protease inhibitors (5 µg/ml leupeptin, 10 µg/ml benzamidine, 10 µg/ml Bacitracin, 5 µg/ml pepstatin, 5 µg/ml aprotinin) and lysed by physical disruption with glass beads. Debris is removed by centrifugation at 1000 X g for 10 min. The membrane fraction is isolated by centrifugation at 100,000 X g for 10 min. This pellet is washed once in lysis buffer plus inhibitors. Polyacrylamide gel electrophoresis of yeast extracts is performed by standard methods except without boiling of samples. Proteins are transferred to Immobilon-P millipore filters by the semi-dry technique. Receptor protein is visualized using ECL reagents with rabbit anti-Ste2 antibodies.

Radioligand binding assays. Reactions are performed in a volume of 0.2 µl with 5 to 50 µg of protein. Binding assays for 5HT1a receptor or β2-adrenergic receptor ligands use buffer of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$. Somatostatin binding is performed in a buffer of 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$. After allowing ligand binding to reach equilibrium at room temperature, membrane fractions are isolated on GFC glass fiber filters. The following final concentrations of ligands are used: radioligands-^{3}H spiperone, 80 nM; ^{125}I-cyanapindolol, 250 pM; [^{125}I-tyr^{11}]-somatostatin 14, 250 pM; competitors-serotonin, 10 µM; propranolol, 20 µM somatostatin 14, 1 µM. The guanosine triphosphate analog Gpp(NH)p is used at 100 µM.

Expression of the human 5HT1a serotonergic receptor. The gene encoding the human 5HT1a receptor is modified to add the first 14 amino acids of the yeast Ste2 protein, cloned into the expression plasmid pMP3 and,

designated pCHI11. This strain, designated CY382, is grown in medium containing galactose to induce receptor expression, fractioned and tested for receptor activity by binding of the radiolabelled antagonist ^{3}H-spiperone. Saturation binding demonstrates that the receptor is expressed at high levels (Bmax = 3.2 pmol/mg protein) and that it binds spiperone with an affinity (Kd = 115 nM; Figure 3) similar to that observed in mammalian tissues (Kd - 20 to 100 nM).

Two chimeric receptor genes are engineered; in pCH117, sequences encoding the N-terminus including the first two transmembrane domains of the 5HT1a receptor are replaced with the corresponding sequences of the Ste2 receptor, and in pCH118, these Ste2 sequences are added directly to the N-terminus of the 5HT1a receptor to create a novel nine-transmembrane-domain receptor (Figure 4). Strains expressing these receptors are examined for binding of radiolabelled ligand. Both receptors demonstrate specific binding of the 5HT receptor antagonist ^{3}H-spiperone (Figure 4). Replacement of the first two transmembrane domains with those of an unrelated receptor does not apparently affect binding of this ligand. Addition of transmembrane domains do not effect binding, suggesting that this unusual receptor can attain a functional conformation in the cell membrane. Strains carrying pCHI11, pCHI17, or pCHI18 produce Bmax values of 3.1, 1.6, or 0.7 pmol/mg, respectively. Although these chimeric receptors produce interesting results regarding receptor structure, they do not enhance overall levels of functional receptors in the cell.

All intracellular sequences of the 5HT1a receptor are replaced with corresponding sequences of the yeast Ste2 protein to directly couple the receptor to the yeast G protein. The resultant chimeric receptor, CHI16, is expressed in a wild-type yeast strain and examined for high

affinity binding of 5HT1a receptor agonists. Agonist binding is not detected. However, the level of radiolabelled spiperone binding is equal to CHI11, indicating that this receptor is expressed at high levels and in a functional conformation.

Expression of the human β2-adrenergic receptor. The human adrenergic receptor is expressed in yeast with the intention of using it as a model to optimize expression and G protein coupling. A yeast strain expressing the receptor is examined by Scatchard analysis for binding of the ligand ^{125}I-cyanopindolol. Binding is saturable and demonstrated a Kd (23 pM), similar to that reported in mammalian tissues. Strains with or without coexpressed Gαs are then examined in competition assays in which binding of this radioligand is competed with the agonists isoproterenol or epinephrine. High affinity binding, which is only expected to occur if the receptor is actively coupled to G protein, was observed in both strains (Figures 5 and 6). The extrapolated Ki values for these ligands (isoproterenol = 10 nM; epinephrine =60 nM) are consistent with affinities observed in mammalian tissues and exhibit the expected order of potency. However, other data suggest that the high affinity binding of agonists to the β2-adrenergic receptor in yeast is anomalous and not a result of coupling to G protein. In particular, the third intracellular loop (containing the primary Gα contact points) of the β2-adrenergic receptor is replaced with the corresponding domain of the yeast Ste2 receptor. This receptor exhibits the same affinities for adrenergic agonists suggesting that the β2-adrenergic receptor takes on an inappropriate conformation in yeast.

Expression of rat somatostatin receptor. High affinity binding of somatostatin to SSTR2 is dependent on formation of a receptor/G protein complex (Strnad et al, 1993). When SSTR2 and G protein are uncoupled from each other, high affinity binding of [^{125}I]tyr^{11}S-14 is attenuated.

As shown in Figure 8, binding of [^{125}I]tyr^{11}S-14 to SSTR2 expressed in yeast coexpressing Scg1/$G_{\alpha i2}$ is saturable and of high affinity. The calculated K_d of [^{125}I]tyr^{11}S-14 binding SSTR2 expressed in yeast is 600 pM. Similar binding affinities are observed when $G_{\alpha i2}$ is coexpressed with SSTR2 rather than Scg1/$G_{\alpha i2}$. The K_d observed in yeast is in close agreement to the calculated K_d of [^{125}I]tyr^{11}S-14 binding of SSTR2 expressed in mammalian cells (Strnad et al., 1993). It is demonstrated that addition of the yeast Ste2 receptor to the N-terminus of this receptor has no affect on its ability to bind S-14. The Ste2 sequences act as a tag for immunochemical examination of receptor expression. The immunoblot shown in Figure 7 illustrates the high level of expression of SSTR2 in three different strains. Yeast strains expressing the SSTR2 somatostatin receptor and different Gα proteins are derived from strain YPH500. These strains share the genotype MATa *scg1Δ hisG lys2-801 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 ade2* SSTR2. Strains designated CY624 (Gαi2), CY602 (Gαs, and CY603 *Scg1*) are examined for specific binding of radiolabelled somatostatin-14 (S-14). All three exhibit some degree of somatostatin binding (Table 1). High affinity binding of this ligand requires coupling of the receptor to G protein, normally Gαi (Luthin D.R. 1993; Strnad J. 1993), so the high level of binding in the absence of Gαi is unexpected. As confirmation that the receptor is coupled to G proteins, binding is examined in the presence of the nonhydrolyzable guanosine triphosphate analog Gpp (NH)p. The addition of this compound eliminates ligand binding (Table 1), demonstrating that the SSTR2 receptor is coupled to G protein. These data demonstrate that a mammalian G protein-coupled receptor can functionally interact with a G protein composed of its favored Gα protein plus yeast Gβ and Gγ subunits and that a heterologous receptor can functionally couple to a G protein entirely composed of yeast subunits.

TABLE 1. Binding of ^{125}I-somatostatin

STRAIN[a]	Gα	Bmax[b]	+Gpp (NH) p[c]
CY624	Gαi	94.5	22%
CY602	Gαs	13.7	0%
CY603	Scgl	24.7	4%

[a]Crude membrane extracts were prepared from yeast strains expressing the rat SSTR2 somatostatin receptor subtype and the indicated Gα protein. The maximal binding of radiolabeled somatostatin 14 was measured as described in the text. [b]Bmax values are given as fmol/mg total protein. [c]The non-hydrolyzable GTP analog Gpp(NH)p was added to samples to uncouple receptor and G protein. Data are presented as percent radioligand bound compared to untreated samples.

Expression of *Drosophila* muscarinic acetylcholine receptor. DNA sequences encoding a *Drosophila* muscarinic acetylcholine receptor (Dm mAChR) are modified by addition of a SalI site in the 5' coding sequences through the use of PCR. DNA sequences encoding the first 23 amino acids of the *STE2* gene product are added to the 5' end of Dm mAChR as a BamHI/SalI fragment. The modified Dm mAChR is inserted into the BamHI site in plasmid pMP3, placing expression of the receptor under the control of the *GAL1* promoter, forming plasmid pMP3-Dm mAChR. Strain CY414 is transformed with this plasmid and cultured for receptor expression by standard methods. Crude membrane preparations are prepared from these cells and tested for the presence of specific binding sites for the muscarinic antagonist 3H-quinuclidinyl benzilate (10 nM) competed with atropine (50 μM). Specific binding sites (Bmax 10 and 30 fmol/mg) are observed.

Drosophila mAchR expression is also detectable by immunoblotting methods. An abundant 75 kDa polypeptide, consistent with the predicted molecular weight from the

primary sequence of mAchR, is detected in samples of protein (30µg/lane) from crude membrane preparations from cells expressing mAchR from pMP3 using an antibody directed against the associated Ste2 epitope (Figure 9). Substantially less protein is detected when mAchR is expressed from pMP2, a derivative of pMP3 lacking *GAL4* sequences, which is not expected to confirm high level expression of mAchR.

Expression of an α2-adrenergic receptor (α2-AR). An EcoRI-NarI fragment from plasmid pMP3, including the *GAL1,10* promoter EcoRI-BamHI fragment, DNA sequences encoding the first 23 amino acids of the STE2 gene product present on a BamHI-SalI fragment, SalI-SphI polylinker fragment from YEp352, and STE7 terminator sequences, is transferred to pRS424, forming pLP15. A PstI-PvuII fragment encoding a porcine α2A-AR [Guyer, C.A., Horstman, D.A., Wilson, A.L., Clark, J.D., Cragoe, E.J., and Limbird, L.E. (1990) J. Biol. Chem. 265, 17307-17317] is inserted into the PstI-SmaI sites of pLP15, forming pLP50. An EcoRI fragment of GAL4 is inserted into the EcoRI site of pLP50, forming pLP60. Strain LY124 [a derivative of YPH500 (Stratagene) containing the *scg1ΔhisG* allele and bearing plasmid pLP10 [pLP10: pUN75 (Elledge, S.J. and Davis, R.W. Genetics 87, 189-194) containing the PGK-Scg1-Gαi2 XhoI-SalI fragment from pPGKH-Scg1-Gαi2 inserted into the SalI site] is transformed with pLP60 and cultured for receptor expression by standard methods. Crude membrane preparations are prepared from these cells and tested for the presence of specific binding sites for the α2-AR antagonist 3H-rauwolscine (200 nM) competed with phentolamine (10µM). Specific binding sites with a Bmax of between 10 and 84 fmol/mg were observed.

Porcine α2AR expression is also detectable by immunoblotting methods (Figure 10). Several abundant polypeptides are detected in samples of protein (30µg/lane)

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from crude membrane preparations from cells expressing α 2AR from pMP3 using an antibody directed against the associated Ste2 epitope.

EXAMPLE 3

Agonist dependent growth of yeast in response to somatotstatin receptor agonists.

Yeast strains that respond to somatostatin are created by introducing several modifications into typical laboratory yeast strains. First, a cDNA encoding the somatostatin receptor subtype 2 (SSTR2) is placed under the control of the galactose-inducible GAL1 promoter in a multicopy yeast plasmid (Figure 11). High level expression of receptor is accomplished by inducible co-overexpression of the transcriptional activating protein GAL4 from the same plasmid. Second, the endogenous Gα protein gene, GPA1/SCG1, is replaced with a chimeric gene composed of an amino terminal domain from GPA1/SCG1, and C-terminal sequences from rat Gαi2 (Figure 12). Expression of chimeric G proteins in yeast have previously been shown to suppress the growth defect of scg1/gpa1 mutant cells [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590]. Third, the FAR1 gene is deleted, permitting continued growth in the presence of an activated mating signal transduction pathway. The FAR1 protein is thought to serve as the primary interface between the mating signal transduction pathway and cell cycle machinery [Chang, F. and Herskowitz, I. (1990) Cell 63, 999-1012; Peter, M., Gartner, A., Horecka, J., Ammerer, G., and Herskowitz, I. (1993) Cell. Biol. 13, 5659-5669]. Fourth, the FUS1 gene is replaced with a reporter gene construct made by fusing transcription control elements of the FUS1 gene to HIS3

protein coding sequences, thereby placing expression of the HIS3 gene product under the control of the pheromone signal transduction pathway. Yeast strains (his3) bearing this construct are able to grow poorly on supplemented minimal medium lacking histidine and are sensitive to 3-amino-1,2,4-triazole (AT), an inhibitor of the HIS3 gene product. Receptor activation by agonist-binding leads to increased HIS3 protein expression, a corresponding increase in resistance to AT, and, therefore, the ability to grow on medium lacking histidine and/or in the presence of the inhibitor. Adjusting the pH of the growth medium to > pH 5.5 enhances the ability of such cells to grow in presence of agonist, presumably due to an increased ability of somatostatin to bind to SSTR2.

The utility of the yeast expression system lies in its adaptability to rapid mass screening. To facilitate screening for novel therapeutics directed at the SSTRs, a convenient agar plate bioassay is developed in which functional coupling of somatostatin binding to receptor and subsequent activation of the mating signal transduction pathway is detected as a zone of growth (halo) around applied compounds (Figure 13). Overnight liquid cultures of LY268 [a derivative of YPH500 (Stratagene) MATa ura3-52 lys2-801 ade2 trp1Δ63 his3Δ200 leu2Δ1 far1ΔLYS2 scg1ΔhisG fus1ΔFUS1-HIS3 sst2ΔADE2 bearing the SSTR2 expression plasmid and the SCG1-Gαi2 expression plasmid pLP82 in SC dextrose (2%) lacking ura, trp were transferred to SC Lactate medium (2%) lacking ura and trp and subsequently SC galactose (2%) medium lacking ura and trp. Cells (2 $x10^5$) are then plated in 30 ml of SC galactose (2%) lacking ura, trp, and his agar (Figure 13, top panel), or spread evenly on the surface (Figure 13, bottom panel), the indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days. Halos of growth are observed around

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disks saturated with varying concentrations of somatostatin (S-14) and MK678 [a hexapeptide analog of somatostatin that exhibits high affinity binding to SSTR2 [Verber, D., Saperstein, R., Nutt, R., Friedinger, R., Brady, P., Curley, P., Perlov, D., Paleveda, W., Zacchei, A., Cordes, E., Anderson, P., and Hirschmann, R. (1981) Life Sci, 35, 1371-1378]. Halo size increases in proportion to the amount of agonist applied and a significant response is observed even at the lowest amount applied (6 nmol S-14), demonstrating the exquisite sensitivity of the assay. No detectable response is observed with carrier alone, or to met-enkephalin, an opiate receptor agonist, demonstrating the high specificity of the assay.

Yeast medium and culture conditions are formulated according to standard procedures and DNA-mediated transformation of yeast is by the LiAc method [Sherman, F., Fink, G.R., and Hicks, J.B. (1986) Methods in Yeast Genetics (Cold Spring Harbor Laboratory Press]. LY268 is constructed by sequential insertional deletion using recombinant scg1:ΔhisG, far1ΔLYS2, FUS1ΔHIS3, and sst2ΔADE2 alleles. The scg1ΔhisG allele is assembled by inserting the hisG-URA3-hisG fragment from pNKY51 [Alani, E., Cao, L., and Kleckner, N. (1987) Genetics 116, 541-545] between the 5' EcoRI-HindIII and 3' SphI-SnaBI fragments of SCG1/GPA1. After DNA-mediated transformation of appropriate yeast strains and selections for replacement of the chromosomal allele, the URA3 gene is removed by inducing recombination between hisG repeats by growth on 5-fluoroorotic acid (FOA)-containing medium [Boeke, J., Lacroute, F., and Fink, G. (1984) Mol. Gen. Genet. 197, 345-346]. The far1ΔLYS2 allele is constructed by amplifying two fragments of the FAR1 gene [Chang, F. and Herskowitz, I. (1990) Cell 63, 999-1012] from yeast genomic DNA (strain YPH501, Stratagene) using synthetic oligonucleotides that introduce an EcoRI site at 1201 in the 5' fragment and an HindIII site at position 2017

and a SalI site at 2821 in the 3' fragment. The fragments are cloned into the EcoRI/SalI fragment of pBSK (Stratagene). The completed far1ΔLYS2 construct is digested with EcoRI and used to transform yeast. An EcoRI fragment encoding the FUS1-HIS3 reporter gene is released from pSL1497 [Stevenson, B.J., Rhodes, N., Errede, B., and Sprague, G.F. (1992) Genes Dev. 6, 1293] and used to transform appropriate yeast strains. The sst2ΔADE2 allele [Dietzel, C. and Kurjan, J. (1987) Mol. Cell. Biol. 7, 4169-4177] is built from a 2.5 kb fragment of the ADE2 gene amplified by PCR using oligos that placed a ClaI site at position 1 and an NheI site at position 2518. This fragment is used to replace the internal ClaI-NheI fragment in SST2. The sst2ΔADE2 fragment is released by digestion with SalI and used to transform appropriate yeast strains.

The multistep construction of the SSTR2 expression plasmid, pJH2 (Figure 11), is initiated by inserting a SphI/NarI fragment of 3' untranslated region from the STE7 gene [Teague, M.A., Chaleff, D.T., and Errede, B. (1986) Proc. Natl. Acad. Sci. USA 83, 7371-7375], and an EcoRI/BamHI fragment of the GAL1/10 promoter [Yocum, R.R., Hanley, S., West, R., and Ptashne, M. (1984) Mol. Cell. Biol. 4 1985-1998] into appropriate sites in YEp352 [Hill, J.E., Myers 2, A.M., Koerner, T.J., and Tzagoloff, (1986) Yeast 2, 163-167.], creating pEK1. A PCR product, encoding the open reading frame and transcriptional termination sequences of the GAL4 gene, is amplified with oligos containing 5' EcoRI and 3' AatII sites and inserted into pEK1, creating pMP3. The cDNA encoding rat SSTR2 (Strnad, J., Eppler, C.M., Corbett, M., and Hadcock, J.R. (1993) BBRC 191, 968-976] is modified by PCR using oligonucleotides that add a BglII site in the DNA sequences encoding the amino terminus of SSTR2 and a BglII site directly after the translational stop site. SSTR2 coding sequences are inserted as a BglII PCR fragment into the BamHI site of pMP3.

Plasmid pLP82 (Figure 12) is constructed by first replacing the XhoI/EcoRI promoter fragment in pPGKH-SCG1-Gαs [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590], with a modified *SCG1* promoter fragment [Dietzel, C. and Kurjan, J. (1987) Cell 50, 1001-1010] amplified from yeast genomic DNA using oligonucleotides that introduce 5' XhoI and 3' EcoRI sites at positions -200 and -42, forming plasmid pLP61. The BamHI fragment encoding Gαs domain is replaced with a comparable fragment encoding Gαi2, forming plasmid pLP71. The XhoI/SalI fragment of pLP71 encoding an *SCG1*-Gαi2 chimeric G protein expressed under the control of the *SCG1* promoter is transferred to the SalI site in pRS414 (Stratagene), forming pLP82.

Plasmid pLP83 is constructed by replacing the EcoRI fragment in pLP71 with the EcoRI fragment encoding I from pPGKH-SCG1 [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590], forming plasmid pLP75. The XhoI/SalI fragment encoding SCG1 is transferred to the SalI site in pRS414 (Stratagene), forming plasmid pLP83.

EXAMPLE 4

Effects of G protein expression on the sensitivity of the bioassay.

Yeast strains LY268 (pLP82: CEN pSCG1-Scg1-Gαi2), LY262 (pRS414-PGK-Scg1-Gαi2: pRS414 containing the PGK-Scg1-Gαi2 Xho/SalI fragment from pPGKH-Scg1-Gαi2 in the SalI site), LY324 (pLP84: 2μ pSCG1-Scg-Gαi2), and LY284 (pRS424-PGK-Scg-Gαi2: pRS424 containing the PGK-Scg1-Gαi2 Xho/SalI fragment from pPGKH-Scg1-Gαi2 in the SalI site) were constructed by placing the designated plasmids in strain LY260 [a derivative of YPH500 (Stratagene) MATa ura 3-52

lys2-801 ade2 trp1D63 his3Δ200 leu2α1 far1ΔLYS2 scg1ΔhisG fus1ΔFUS1-HIS3 sst2ΔADE2 bearing the SSTR2 expression plasmid]. Overnight liquid cultures in SC-Dextrose (2%) lacking ura and trp were transferred to Sc-Lactate (2%) medium lacking ura and trp and subsequently SC-Galactose (2%) medium lacking ura and trp. Cells ($2x10^5$) are then plated in 30 ml of SC-Galactose (2%) lacking ura, trp, and his agar, the indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days (Figure 14). The extent of growth around S-14 is dependent upon the G protein expression plasmid contained in each strain. The most luxuriant growth is observed in response to S-14 by LY268 (pLP82: CEN pSCG1-Scg1-Gαi2), less growth is seen in strains LY262 (pRS414-PGK-Scg1-Gαi2) and LY324 (pLP84: 2μ pSCG1-Scg-Gαi2), while little detectable growth is exhibited by LY284 (pRS424-PGK-Scg1-Gαi2). These results are consistent with the observed inhibition of pheromone stimulated transcriptional induction by elevated amounts of expressed Gα protein. Thus, precise regulation of G protein expression levels in strains expressing heterologous G protein-coupled receptors is critical to the design and successful implementation of a bioassay for compounds that interact with the receptor.

Plasmid pLP84 is constructed by first replacing the XhoI/EcoRI promoter fragment in pPGKH-SCG1-$G\alpha_s$ [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10, 2582-2590], with a modified *SCG1* promoter fragment [Dietzel, C. and Kurjan, J. (1987) Cell 50, 1001-1010] amplified from yeast genomic DNA using oligonucleotides that introduce 5' XhoI and 3' EcoRI sites at positions -200 and -42, forming plasmid pLP61. The BamHI fragment encoding $G\alpha_s$ domain is replaced with a comparable fragment encoging $G\alpha_{i2}$, forming plasmid pLP71. The XhoI/SalI fragment of pLP71 encoding a *SCG1*-$G\alpha_{i2}$ chimeric G protein

expressed under the control of the SCG1 promoter is transferred to the SalI site in pRS424 (Stratagene), forming pLP84.

EXAMPLE 5

Somatostatin receptor is capable of transmitting signal through the endogenous yeast G α

SSTR2 is thought to couple to $G\alpha_{i2}$ and $G\alpha_{i3}$ in mammalian cells [Luthin, D. R., Eppler, C. M., and Linden, J. (1993) J. Biol. Chem. 268, 5990-5996]. However, when expressed in appropriate yeast strains (described below), SSTR2 is shown to be capable of transmitting a signal through the endogenous yeast Gα protein. Implicit in this observation is the necessary coupling of SSTR2 to the endogenous Gα protein. The ability of heterologous G protein-coupled receptors to couple to the endogenous Gα protein is a significant improvement in existing technology, and is thought not to be possible in the prior art (King K. Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J. (1990) Science 250, 121-123). Yeast strains LY266 (pLP83: CEN pSCG1-Scg1), LY280 (pRS414-PGK-Scg1: pRS414 containing the PGK-Scg1 Xho-SalI fragment from pPGKH-ScgI in the SalI site), LY326 (pLP86: 2μ p SCG1-Scg), and LY282 (pRS424-pPGK-Scg pRS424 containing the PGK-Scg1 Xho-SalI fragment from pPGKH-Scg1 in the SalI site) were constructed by placing the designated plasmids in strain LY260. Overnight liquid cultures of these strains, which are capable of expressing only *SCG1/GPA1*, in SC-Dextrose (2%) lacking ura and trp were transferred to SC-Lactate (2%) lacking ura, trp, and subsequently to SC-Galactose (2%) lacking ura and trp medium. Cells (2×10^5) are then plated in 30 ml SC-Galactose (2%) lacking ura, trp, and his medium, the

indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days. Halos of growth are observed around disks saturated with varying concentrations of S-14, demonstrating that a productive signal can be transduced through an interaction between SSTR2 and the yeast *SCG1/GPA1* protein (Figure 15). These observations demonstrate that a simple and broadly applicable bioassay may be established in which any member of the class of G protein-coupled receptors may be expressed in appropriately modified yeast strains and made to couple to the endogenous Gα protein.

Plasmid pLP86 is constructed by replacing the EcoRI fragment in pLP71 with the EcoRI fragment encoding SCG1 from pPGKH-SCG1 [Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J.M., and Tipper, D.J. (1990) Mol. Cell. Biol. 10. 2582-2590], forming plasmid pLP75. The XhoI/SalI fragment encoding SCG1 is transferred to the SalI site in pRS424 (Stratagene), forming plasmid pLP86.

EXAMPLE 6

Mutations in SST2 enhance the sensitivity of the responses to somatostatin

Mutations in the SST2 gene result in supersensitivity of otherwise wild type cells to mating pheromone. The effect of this mutation on levels of AT resistance expressed in far1, FUS1-HIS3 strains is examined (Figure 16). Cells (5×10^5) from overnight cultures of LY230 [a derivative of YPH50 (Statagene) MATa SST2 ura3-52 lys2-801 ade2 trp1Δ63 his3Δ200 leu2Δ1 far1ΔFUS1-HIS3] and LY238 (a modification of LY230 sst2ΔADE2) in SCD-ura, trp are plated on SCD-ura, trp, his, containing 10 mM AT and incubated at 30°C for 2 days. Increased AT resistance in

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response to mating factor is exhibited by LY238 (sst2). Growth of LY238 is observed around disks containing 10 pmol of mating pheromone, while LY230 (SST2) required 1 nmol of mating factor for significant growth to be observed. Thus, introduction of sst2 into these strains raises the sensitivity of the bioassay by at least 100-fold, opening the possibility of increasing the sensitivity of other G protein-coupled receptor bioassays as well.

Yeast strains that express SSTR2 and bearing a defective sst2 gene exhibit much greater growth around disks containing various concentrations of somatostatin than is exhibited by strains containing a functional SST2 gene. Overnight cultures of strains LY268 (sst2, containing pLP82), LY266 (sst2, containing pLP83), LY288 [a derivative of YPH500 (Stratagene) MATa SST2 ura3-52 lys2-801 ade2 trp1Δ63 his3Δ200 leu2Δ1 farlΔLYS2 scg1ΔhisG fus1ΔFUS1-HIS3 bearing the SSTR2 expression plasmid and the SCG1-Gα_{12} expression plasmid, pLP82] and LY290 (a modification of LY288 that contains pLP83) in SC Dextrose (2%) lacking ura, trp were transferred to SC Lactate medium (2%) lacking ura and trp, and subsequently to SC Galactose (2%) medium lacking ura and trp. Cells (2 x 10^5) are then plated in 30 ml SC Galactose (2%) plates lacking ura, trp and his, the indicated amounts of selected compounds applied to paper disks situated on the surface of the agar plate, and incubated at 30°C for 3-5 days. Halos of growth are observed around disks saturated with varying concentrations of S-14 in both sst2Δ and SST2 strains (Figure 17), however, the amount of growth exhibited by strain LY268 and LY266 (sst2) is significantly greater than that observed for strains LY288 and LY290 (SST2). Furthermore, the down-regulatory effect of SST2 is more pronounced in those strains that express solely the wild type SCG1/GPA1 protein, consistent with the expectation that SST2 interacts more faithfully with the native protein than the SCG1-Gα_{12}

chimeric protein.

EXAMPLE 7

Functional expression of a rat cholecystokinin (CCK_B) receptor in yeast.

Cholecystokinin (CCK) is a major intestinal hormone that plays an important role in regulating pancreatic secretion and bile ejection (1). CCK is also one of the most widely distributed of brain neuropeptides (2). CCK promotes its effects through the action of cell surface receptors which can be classified using pharmacological criteria into two subtypes, CCK_A and CCK_B (3). Molecular cloning efforts have identified cDNAs encoding G protein-coupled CCK_A (4) and CCK_B (5-8) receptors. Recently, compounds with selective CCK_B receptor antagonist properties having potent anxiolytic activity have been identified (9). Functional expression of CCK_B receptors in yeast should permit rapid screening for new compounds with CCK_B antagonist properties and facilitate molecular characterization of structural aspects of the CCK_B receptor required for rational design of new CCK_B ligands.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (10). The rat CCK_B receptor was cloned from rat brain cDNA by PCR using oligonucleotide primers that introduce BglII sites at 5' and 3' ends (5-AAAAGATCTAAAATGGACCTGCTCAAGCTG, 3' AAAAGATCTTCAGCCAGG CCCCAGTGTGCT). The CCK_B receptor expression plasmid, pJH20,

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was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (11). The Gα protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of GPA1 in pLP83 (11) with those of $G\alpha_s$(pLP122), $G\alpha_{i2}$ (pLP121).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (12). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express the rat CCK_B receptor were constructed by sequential DNA-mediated transformation of LY296 (MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2, ref. 7) with pJH20 followed by the Gα protein expression plasmids described above.

Radiolabeled agonist saturation binding assays. Crude yeast membrane extracts from late log phase cultures were prepared by glass-bead lysis and centrifugation at 40,000 xg following a published procedure (13). The protein content of crude membrane fractions was measured using the Biorad protein assay kit according to manufacturers instructions. Radioligand binding assays were conducted according to Strnad et al (14) using ^{3}H-CCK-8 (Amersham) in the presence of 150 mM NaCl. Non-specific binding was defined as that observed in the presence of 1 μM CCK-4. Negligible specific binding was observed in membrane fractions made from cells lacking CCK_B receptor (data not shown).

Bioassay. Functional assay of CCK_B receptor expressed in yeast was accomplished using modification of a standard procedure (11). Yeast strains were grown overnight

in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (50°C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 x10^4 cells/ml) and poured into square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Cholecystokinins (CCK-4, CCK-8), somatostatin (S-14), and met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

Cholecystokinin binding to the rat CCK $_B$ receptor expressed in yeast. High level functional expression of the rat CCK_B receptor in yeast was a necessary prerequisite to the development of a useful bioassay. The rat CCK_B receptor cDNA was placed under the control of the GAL1 promoter in plasmid pJH20. This construct also confers inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking GAL4 sequences (data not shown). CCK_B receptor sequences were introduced into pJH20 without modification of the protein coding sequences. Previously, King et al. reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent STE2 sequence was necessary for efficient receptor expression in yeast (15). In contrast, functional expression of CCK_B

receptor in yeast does not require addition of any yeast sequences to the amino-terminus. Plasmids conferring expression of chimeric Gα proteins composed of amino-terminal βγ-interaction domain from Gpalp and carboxy-terminal receptor interaction domains from rat $G\alpha_{i2}$ (pLP121) or $G\alpha_s$ (pLP122) under the control of the *GPA1* promoter were constructed. Yeast strains that contain expressed CCK_B receptor and chimeric $G\alpha_{i2}$ (LY628) and $G\alpha_s$ (LY631) protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with CCK_B receptor and Gα protein expression plasmids. Most G protein-coupled receptors exhibit both high and low agonist-dependent affinity states. High-affinity agonist binding is dependent on functional association of receptor with a heterotrimeric G protein. If the receptor does not associate with, or is uncoupled from the G protein, agonist binding will be of low affinity and undetectable in radiolabeled agonist saturation binding assays. In crude yeast membrane fractions made from LY631 cells, the agonist ^{3}H-CCK-8 bound to the CCK_B receptor with high affinity and in a saturable manner (Fig. 18), demonstrating that (1) a functional ligand-binding conformation of the CCK_B receptor was expressed in yeast, and (2) the receptor functionally associated with the chimeric Gα protein, resulting in a high-affinity agonist binding state. The CCK_B receptor expressed in yeast displayed an affinity for ^{3}H-CCK-8 (K_d = 8 nM) substantially lower than the high affinity binding state of the CCK_B receptor expressed in mammalian cells (K_d = 100 pM, ref. 5), perhaps due to an inefficient interaction with the receptor interaction domain from rat $G\alpha_s$ These binding parameters would be expected to more closely resemble the native values in extracts from cells containing the cognate Gα protein, Gαq (5-8). The total number of ^{3}H-CCK-8 binding sites observed (B_{max}=206 fmol/mg) was consistent with values

obtained for the yeast α-mating pheromone receptor (200 fmol/mg, ref. 16). For many G protein-coupled receptors, high affinity agonist binding is sensitive to GTP and its analogs. GTP analogs induce dissociation of the receptor/G-protein complex, resulting in a low affinity agonist binding state. Addition of GppNHp (100 μM), a non-hydrolyzable GTP analog, to an agonist binding assay decreased specific binding of ^{3}H-CCK-8 to the CCK_B receptor by greater than 50% in crude membrane fractions from LY631 cells. These results represent a further indication of functional coupling between the CCK_B receptor and the chimeric Gα protein. Thus, the rat CCK_B receptor expressed in yeast exhibits high-affinity agonist binding properties comparable to those observed in mammalian tissues.

The CCK_B receptor retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using yeast strains bearing the above described genetic modifications and plasmids conferring expression of the CCK_B receptor and chimeric $G_{\alpha i2}$ and $G_{\alpha s}$ proteins. A dose-dependent growth response of LY628 and LY631 cells was evident around applied CCK-4 (Fig. 19). The assay was selective: the diameter of the growth zones was proportional to the reported affinity of the ligands for the CCK_B receptor (CCK-8 > gastrin > CCK-4) reflecting the ability of the bioassay to discriminate between ligands of varying potency (5-8). Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (somatostatin, met-enkephalin, oxymetazoline, isoproterenol, carbachol), nor by yeast cells lacking the CCK_B receptor (data not shown). A detectable response was observed to 10 μg of CCK-4.

DISCUSSION

Compounds that act at the CCK receptors, particularly

antagonists, may possess great therapeutic potential (3). In the periphery, the inhibitory effects of CCK antagonists make them excellent candidates for treatment of pancreatitis, pancreatic cancer, biliary colic, disorders of gastric emptying, and irritable bowel syndrome. CCK antagonists reverse the development of satiety and might be useful in improving appetite in anorectic patients or others that require increased food intake. Conversely, CCK agonists might be useful appetite suppressants. CCK antagonists also potentiate opiate analgesia and so might be appropriate for use in the management of clinical pain. In the CNS, selective CCK antagonists have promise as powerful anxiolytic agents (9). Further, CCK antagonists relieve the anxiety associated with withdrawal from drug use, and so might find a use in the treatment of withdrawal from commonly abused drugs. CCK agonists may have use as antipsychotic agents.

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EXAMPLE 9

Functional expression of a rat adenosine (A_{2a}) receptor in yeast.

Adenosine, as well as ATP and related purinergic compounds, function as both neurohormonal agents and autocoids regulating the process of cell to cell communication (1). In this role, adenosine regulates a broad range of physiological functions including heart rate and contractility, smooth muscle tone, sedation, release of neurotransmitters, platelet function, lipolysis, kidney and white blood cell action. Adenosine promotes its effects through the action of cell surface receptors which can be classified using pharmacological criteria into three subtypes, A_1, A_{2a} and A_{2b}, and A_3. Molecular cloning efforts have identified cDNAs encoding G protein-coupled adenosine A_1 (2-5), A_{2a} and A_{2b} (6-9), and A_3 receptors (10). Functional expression of adenosine receptors in yeast should permit

rapid screening for new compounds with adenosine agonist and antagonist properties and facilitate molecular characterization of structural aspects of the adenosine receptors required for rational design of new adenosine ligands.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (11). The rat A_{2a}-adenosine receptor (9) was cloned from rat brain cDNA by PCR using oligonucleotide primers that introduce BamHI sites at 5' and 3' ends (5' GAAGATCTAAAAAATGGGCTCCTCGGTGTAC, 3' ACATGCATGCAGATCTTCAGGAAGGGGCAAACTC). The A_{2a}-adenosine receptor expression plasmid, pJH21, was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (12). For constitutive expression of the A_{2a}-adenosine receptor in glucose-containing medium, the expression vector, pLP100, was constructed. DNA fragments encoding transcriptional regulatory sequences from the *ADH1* gene (&) were amplified by PCR and inserted into pRS426. An ADH1 transcriptional terminator fragment was amplified from yeast genomic DNA (YPH501, Stratagene) using synthetic oligonucleotides that add 5' XhoI (TTTCTCGAGCGAATTTCTTATGATTT) and 3' KpnI (TTTGGTACCGGGCCCGGACGGATTACAACAGGT) sites. An *ADH1* promoter fragment was amplified from yeast genomic DNA using synthetic oligonucleotides that add 5' SacI GGGAGCTCTGATGGTGGTACATAACG) and 3' BamHI (GGGGGATCCTGTATATGAGATAGTTGA) sites. The A_{2a}-adenosine receptor expression plasmid, pLP116, was constructed by inserting a PCR fragment encoding the A_{2a}-adenosine receptor amplified using oligonucleotides that add 5' BglII (AAAGATCTAAAATGGGCTCCTCGGTGTAC) and 3' SalI (AAGTCGACTCAGGAA

GGGGCAAACTC) sites BamHI-SalI cut LP100. The Gα protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of *GPA1* in pLP83 (12) with those of $G_{\alpha s}$ (pLP122) and $G\alpha_{i2}$ (pLP121).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (13). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequentional insertional deletion using recombinant alleles. Yeast strains that express the rat A_{2a}-adenosine receptor were constructed by sequential DNA-mediated transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔAΔE2* , ref. 12) with A_{2a}-adenosine receptor expression plasmids followed by the Gα protein expression plasmids described above.

Radiolabeled agonist saturation binding assays. Crude yeast membrane extracts from late log phase cultures were prepared by glass-bead lysis and centrifugation at 40,000 xg following a published procedure (14). The protein content of crude membrane fractions was measured using the Biorad protein assay kit according to manufacturers instructions. Radioligand binding assays were conducted according to Strnad *et al.* (15) using ^{3}H-NECA (Amersham). Non-specific binding was defined as that observed in the presence of 1 μM NECA. Negligible specific binding was observed in membrane fractions made from cells lacking A_{2a}-adenosine receptor (data not shown).

Bioassay. Functional assay of the A_{2a}-adenosine receptor expressed in yeast was accomplished using a modification of a standard procedure (12). Yeast strains were grown overnight in 2 ml synthetic complete liquid

medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (50°C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) containing 5 mM 3-aminotriazole (Sigma) was inoculated with the overnight culture (2 $x10^4$ cells/ml) and plated in square (9 x 9 cm) petri plates. For expression of the A_{2a}-adenosine receptor in glucose-containing medium, samples were removed from the first overnight culture and assayed in agar medium composed as above with glucose (2%) replacing galactose. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of DMSO containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Adenosine ligands CGS-21680, NECA, and DPMA were purchased from RBI. Somatostatin (S-14) and met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

Adenosine agonist binding to the rat A_{2a}-adenosine receptor expressed in yeast. High level functional expression of the A_{2a}-adenosine receptor in yeast was a necessary prerequisite to the development of a useful bioassay. In plasmid pJH21, the rat A_{2a}-adenosine receptor cDNA was placed under the control of the inducible *GAL1* promoter. This construct also confers inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). Plasmid pLP116 confers high level constitutive expression of the A_{2a}-

adenosine receptor under the control of the *ADH1* promoter. In both plasmids, DNA sequences encoding the A_{2a}-adenosine receptor were introduced without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast (16). In contrast, functional expression of the A_{2a}-adenosine receptor in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed amino-terminal $\beta\gamma$-interaction domain from Gpa1p and a carboxy-terminal receptor interaction domain from rat $G\alpha_s$ (pLP122) under the control of the *GPA1* promoter was constructed. Yeast strains that contain expressed A_{2a}-adenosine receptor and chimeric Gα protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with A_{2a}-adenosine receptor and Gα protein expression plasmids.

Most G protein-coupled receptors exhibit both high and low agonist-dependent affinity states. High-affinity agonist binding is dependent on functional association of receptor with a heterotrimeric G protein. If the receptor does not associate with, or is uncoupled from the G protein, agonist binding will be of low affinity and undetectable in radiolabeled agonist saturation binding assays. In crude yeast membrane fractions from cells bearing pLP116 and pLP122 (LY626), the agonist ^{3}H-NECA bound to the A_{2a}-adenosine receptor with high affinity and in a saturable manner, (Fig. 20) demonstrating that (1) a functional ligand-binding conformation of the A_{2a}-adenosine receptor was expressed in yeast, and (2) the receptor functionally associated with the chimeric Gα protein, resulting in a high-affinity agonist binding state. The total number of ^{3}H-

NECA binding sites observed (B_{max}=242 fmol/mg) exceeded values obtained for the yeast α-mating pheromone receptor (200 fmol/mg, ref. 17). The affinity of [^{3}H] NECA for the A_{2a}-adenosine receptor in yeast membranes (Kd-8μM) is equivalent to that observed in mammalian cells indicating functional coupling between receptor and G protein.

The A_{2a}-adenosine receptor retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using a yeast strain (LY595) bearing the above described genetic modifications and plasmids conferring expression of the A_{2a}-adenosine receptor (pLP116) and *GPA1* (pLP83). A dose-dependent growth response of LY595 cells was evident around applied CGS-21680, an A_{2a}-adenosine receptor selective agonist. The growth response was significantly more robust than that exhibited by cells responding to NECA and DPMA (Fig. 21). The assay was selective: the diameter of the growth zones was proportional to the reported affinity of the ligands for the A_{2a}-adenosine receptor (CGS-21680>NECA=DPMA) reflecting the ability of the bioassay to discriminate between ligands of varying potency. Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (somatostatin, met-enkephalin, oxymetazoline, isoproterenol, carbachol), nor by yeast cells lacking the A_{2a}-adenosine receptor (data not shown). A detectable response was observed to 10 μg of CGS-21680.

DISCUSSION

Multiple therapeutic opportunities exist for compounds that modulate the function of the adenosine receptors (1). Adenosine agonists may be useful in the treatment of epileptic seizure episodes and in preventing neuronal damage in stroke and neurodegenerative disorders. The antidysrhythmic action adenosine suggests that adenosine

agonists could be effective in the treatment of complex tachycardia. A_2 adenosine agonists have potent sedative, anticonvulsant and anxiolytic activity. A_{2a}-adenosine selective agonists may be useful in stimulating lipolysis in adipose tissue, making them useful as weight loss treatments or antidiabetic agents and in the improvement of carcass quality in agricultural animals. A_1 adenosine antagonists may be useful in treatment of acute renal dysfunction. A_3-antagonists may be useful in modulating mast cell degranulation for treatment of inflamatory disorders, including asthema.

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EXAMPLE 8

Functional expression of a rat somatostatin subtype 5 receptor in yeast.

The cyclic tetradecapeptide somatostatin is a potent inhibitor of secretion of several hormones, including growth hormone from the pituitary, glucagon and insulin from the pancreas, and gastrin from the gut. Somatostatin also acts as a neurotransmitter and has been shown to have broad modulatory effects in CNS and peripheral tissues (1). The effects of somatostatin are transduced through binding of the hormone to high-affinity, plasma membrane localized somatostatin (SSTR) receptors (2). The SSTR's, encoded in five distinct subtypes (SSTR1-5), which account in part for tissue-specific differences in responses to somatostatin (3-10), comprise a subfamily of the seven-transmembrane domain, G protein-coupled receptor superfamily that mediates responses to a broad variety of extracellular signals. Functional expression of SSTR5 in yeast should permit rapid screening for new subtype-selective somatostatin

agonists and compounds with antagonist properties and facilitate molecular characterization of structural aspects of the SSTR5 required for rational design of new somatostatin ligands.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (11). The rat SSTR5 (7) was cloned from rat genomic DNA by PCR using oligonucleotide primers that introduce BglII sites at 5' and 3' ends (5'AAAAAGATCTAAAATGGAGCCCCTCTCTCTG, 3' AGCAGATCTTCAGATC CCAGAAGACAAC). The SSTR5 expression plasmid, pJH19, was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (12). The Gα protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of *GPA1* in pLP83 (12) with those of $G\alpha_s$ (pLP122), $G\alpha_{i2}$ (pLP121).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (13). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express SSTR5 were constructed by sequential DNA-mediated transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, ref. 12) with pJH19 followed by the Gα protein expression plasmids described above.

Bioassay. Functional assay of SSTR5 expressed in yeast

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was accomplished using modification of a standard procedure (12). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-his agar medium (30 µl, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 $x10^4$ cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Somatostatin (S-14, S-28), met-enkephalin, and CCK-8 were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

Somatostatin dependent growth response of yeast cells expressing the SSTR5. High level functional expression of the SSTR5 in yeast was a necessary prerequisite to the development of a useful bioassay. The SSTR5 cDNA was placed under the control of the *GAL1* promoter in plasmid pJH19. This construct also confers inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). SSTR5 sequences were introduced into pJH19 without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient

receptor expression in yeast (15). In contrast, functional expression of SSTR5 in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed amino-terminal β_2-interaction domain from Gpa1p and a carboxy-terminal receptor interaction domain from rat $G\alpha_{12}$ (pLP121) under the control of the *GPA1* promoter was constructed. A yeast strain (LY620) that contains expressed SSTR5 and chimeric Gα protein was assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with SSTR5 (pJH19) and Gα protein expression (pLP121) plasmids. A dose-dependent growth response of LY620 cells was evident around applied S-14 (Fig. 22). Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (CCK-8, met-enkephalin, oxymetazoline, isoproteranol, carbachol), nor by yeast cells lacking the SSTR5 (data not shown). A detectable response was observed to 60 nmol of S-14.

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Example 10

Functional expression of the porcine somatostatin subtype 2 (SSTR2) receptor in yeast.

The cyclic tetradecapeptide somatostatin is a potent inhibitor of secretion of several hormones, including growth hormone from the pituitary, glucagon and insulin from the pancreas, and gastrin from the gut. Somatostatin also acts as a neurotransmitter and has been shown to have broad modulatory effects in CNS and peripheral tissues (1). The effects of somatostatin are transduced through binding of the hormone to high-affinity, plasma membrane localized somatostatin (SSTR) receptors (2). The SSTR's, encoded in five distinct

subtypes (SSTR1-5), which account in part for tissue-specific differences in responses to somatostatin (3-11), comprise a subfamily of the seven-transmembrane domain, G protein-coupled receptor superfamily that mediates responses to a broad variety of extracellular signals. Functional expression of porcine SSTR2 in yeast should permit rapid screening for new species and subtype-selective somatostatin agonists and compounds with antagonist properties and facilitate molecular characterization of structural aspects of the porcine SSTR2 required for rational design of new somatostatin ligands. Compounds identified in high-throughput, mechanism-based screens represent leads for new growth-enhancing agents for use in pigs.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (12). The porcine SSTR2 (11) was cloned from a human brain cDNA library by PCR using oligonucleotide primers that introduce BglII sites at 5' and 3' ends (5' AAAAGATCTAAAATGTCCATTCCATTTGAC, 3' AAAAGGTACCAGATCTTCAGATACTGGTTTGGAG). The porcine SSTR2 expression plasmid, pJH18, was constructed by inserting the BglII-digested PCR fragment in the correct orientation into BamHI cut pMP3 (13).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (14). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express porcine SSTR2 were constructed by sequential DNA-mediated

transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2* , ref. 13) with the chimeric G_{a12} protein expression plasmid, pLP82 (13), followed by pJH18 or pJH17.

Radiolabeled agonist saturation binding assays. Crude yeast membrane extracts from late log phase cultures were prepared by glass-bead lysis and centrifugation at 40,000 xg following a published procedure (15). The protein content of crude membrane fractions was measured using the Biorad protein assay kit according to manufacturers instructions. Radioligand binding assays were conducted according to Strnad *et al* (10) using radiolabeled somatostatin (^{125}I-tyr^{11}-S-14, Amersham). Non-specific binding was defined as that observed in the presence of 1 μM S-14. Negligible specific binding was observed in membrane fractions made from cells lacking porcine SSTR2 (data not shown).

Bioassay. Functional assay of the porcine SSTR2 expressed in yeast was accomplished using modification of a standard procedure (13). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and tryptophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 x10^4 cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 μl of sterile water containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Somatostatin (S-14, S-28), met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol

were from Sigma. MK678 and sandostatin were prepared synthetically.

RESULTS

Somatostatin binding to the porcine SSTR2 expressed in yeast. High level functional expression of the porcine SSTR2 in yeast was a necessary prerequisite to the development of a useful bioassay. The porcine SSTR2 cDNA was placed under the control of the *GAL1* promoter in plasmid pJH17 and 18. These constructs also confer inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). The porcine SSTR2 sequences were introduced into pJH18 without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast (16). In contrast, functional expression of porcine SSTR2 in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed amino-terminal $\beta\gamma$-interaction domain from Gpa1p and a carboxy-terminal receptor interaction domain from rat $G\alpha_{i2}$ (pLP82) under the control of the *GPA1* promoter was constructed. Yeast strains that contain expressed porcine SSTR2 and chimeric G_α protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with porcine SSTR2 (pJH17, pJH18) and G_α protein expression (pLP82) plasmids.

Most G protein-coupled receptors exhibit both high and low agonist-dependent affinity states. High-

affinity agonist binding is dependent on functional association of receptor with a heterotrimeric G protein. If the receptor does not associate with, or is uncoupled from the G protein, agonist binding will be of low affinity and undetectable in radiolabeled agonist saturation binding assays. In crude yeast membrane fractions from cells bearing pJH17, the agonist ^{125}I-tyr^{11}-S-14 bound to the porcine SSTR2 with high affinity and in a saturable manner, demonstrating that (1) a functional ligand-binding conformation of the porcine SSTR2 was expressed in yeast, and (2) the receptor functionally associated with the chimeric Gα protein, resulting in a high-affinity agonist binding state. The total number of ^{125}I-tyr^{11}-S-14 binding sites observed (B_{max}=146 fmol/mg) was consistent with values obtained for the yeast α-mating pheromone receptor (200 fmol/mg, ref. 17).

The porcine SSTR2 retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using a yeast strain (LY474) bearing the above described genetic modifications and plasmids conferring expression of the porcine SSTR2 (pJH18) and a Gpa1-Gα_{i2} chimeric protein (pLP82). A dose-dependent growth response of LY474 cells was evident around applied S-14, MK678, and sandostatin (Fig. 23). The assay was selective: the diameter of the growth zones was proportional to the reported affinity of the ligands for the porcine SSTR2 (S-14=MK678>sandostatin) reflecting the ability of the bioassay to discriminate between ligands of varying potency (18). Detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (met-enkephalin, oxymetazoline, isoproterenol, carbachol), nor by yeast cells lacking the porcine SSTR2 (data not shown). A detectable response was observed to as little as 60 pmol of S-14, illustrating the exquisite sensitivity of the bioassay.

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EXAMPLE 11

Deletion of *MSG5* increases the sensitivity of response to agonist.

The responsiveness of a signal transduction system to a persistent stimulus diminishes with time. This phenomenon, known as desensitization or adaptation, is a universal characteristic of signal response systems. Several molecular mechanisms for adaptation have been described for the yeast mating signal transduction pathway (1). Mutations in the *SST2* gene confer defects in adaptation and increased mating pheromone sensitivity (2,3). The response to applied somatostatin by yeast cells that functionally express the rat SSTR2 is greatly increased in *sst2* mutant cells (4). Mutations in others genes whose products play a role in the adaptation response would be expected to have similar effects. Mutations in the *MSG5* gene, which encodes a putative protein tyrosine phosphatase, cause increased sensitivity to mating pheromone (5). In this study, deletion of *MSG5* in cells that express the rat SSTR2 greatly increases sensitivity to somatostatin. The effect of the *MSG5* mutation is additive with an *SST2* deletion mutation. The double mutant *sst2 msg5* cells form the basis of an extremely sensitive bioassay for compounds that interact with G protein-coupled receptors and G proteins.

MATERIALS AND METHODS

Strain constructions. All molecular biological

manipulations were performed according to standard procedures (6). Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (7). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used in these experiments were constructed using the recombinant *msg5ΔLEU2* allele in pS/PDel and multicopy YEp*MSG5* (5). Yeast strains bearing altered MSG5 levels were constructed by DNA-mediated transformation of LY268 (*MATa ura3-52 trp1D63 his3D200 leu2D1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, pJH2, pLP82) yielding MPY459 (LY268 *msg5ΔLEU2 sst2ΔADE2*) and MPY467 (LY268 YEp*MSG5*) and LY288 (LY268 *SST2*) yielding MPY458 (LY288 *msg5ΔLEU2 SST2*) and MPY466 (LY288 YEp*MSG5*) (4).

Bioassay. Functional bioassay of the rat SSTR2 expressed in yeast was accomplished using modification of a standard procedure (4). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil, tryptophan and leucine (SCD-ura-trp-leu) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp-leu liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-leu-his agar medium (35 ml, adjusted to pH 6.8 by addition of concentrated NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 x10^4 cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the somatostatin (S-14). Plates were incubated at 30 °C for 3 days. Somatostatin (S-14) was from Bachem.

RESULTS

Deletion of *MSG5* promotes increased sensitivity

to ligand. The effects of alterations in the expression of the *MSG5* gene product were assessed by comparing the growth response to S-14 by cells that express the rat SSTR2 (Fig. 24). Mutations that abolish *MSG5* adaptation function would be expected to increase the sensitivity of the bioassay to applied S-14, while overexpression of *MSG5* should blunt the growth response. As expected, a dose-dependent growth response to applied S-14 was observed for LY288 (*SST2 MSG5*). Consistent with expectations, deletion of the *MSG5* gene in MPY458 causes a substantial improvement in sensitivity of the bioassay. The growth response of MPY458 is comparable to that exhibited by LY268 (*sst2ΔADE2, MSG5*). The effects of mutations in both genes was observed in the double mutant MPY459 (*msg5ΔLEU2 sst2ΔADE2*) which showed a further improvement in the growth response. Overexpression of *MSG5* in *SST2* (MPY466) and *sst2ΔADE2* (MPY467) strains severely reduced the growth response.

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EXAMPLE 12.

Functional expression of a human growth hormone release factor (GRF) receptor in yeast.

The growth hormone release factor (GRF) is a potent stimulator of secretion of growth hormone from the pituitary (1). The effects of GRF are transduced through binding of the hormone to high-affinity, plasma membrane localized GRF receptors. The GRF receptor and related secretin-class receptors comprise a subfamily of the seven-transmembrane domain, G protein-coupled receptor superfamily that mediates responses to a broad variety of extracellular signals, and are distinguished by the presence of a large amino-terminal ligand-binding domain (2). Functional expression of the human GRF receptor (3) in yeast should permit rapid screening for new species-selective agonists and facilitate molecular characterization of structural aspects of the GRF receptor required for rational design of new GRF receptor ligands. GRF agonists represent a new class of growth promoting agents for use in agricultural animals and may

find human therapeutic application in the management of growth of children of short stature.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological manipulations were performed according to standard procedures (4). The human GRF receptor (3) was cloned from a human brain cDNA library by PCR using oligonucleotide primers that introduce BamHI sites at 5' and 3' ends (5' ATAGGATCCAAAATGGACCGCCGGATGTGGGGG, 3' ATATGGATCCCTAGCACATAGATGTCAG). The GRF receptor expression plasmid, pJH25, was constructed by inserting the BamHI-digested PCR fragment in the correct orientation into BamHI cut pMP3 (5). The G_a protein expression plasmids used in this study were constructed by replacing DNA sequences encoding the 47 carboxy-terminal amino acids of *GPA1* in pLP83 (12) with those of $G\alpha_s$ (pLP122).

Strain constructions. Yeast strains were constructed, and growth media and culture conditions formulated according to standard procedures (6). DNA-mediated transformation of yeast was carried out using the lithium acetate method. The yeast strains used as the basis for all experiments described in this report were constructed by sequential insertional deletion using recombinant alleles. Yeast strains that express human GRF receptor were constructed by sequential DNA-mediated transformation of LY296 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, ref. 5) with pJH25, followed by the chimeric G_{as} protein expression plasmid, pLP122 (5).

Bioassay. Functional assay of the human GRF receptor expressed in yeast was accomplished using modification of a standard procedure (5). Yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and

tryptophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55 °C) SC Galactose (2 %)-ura-trp-his agar medium (30 ml, adjusted to pH 6.8 by addition of concentrated KOH or NH_4OH prior to autoclaving) was inoculated with the overnight culture (2 $x10^4$ cells/ml) and plated in square (9 x 9 cm) petri plates. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of the designated compounds. Plates were incubated at 30 °C for 3 days. Human GRF (hGRF (1-29)-NH_2), (D-ala^2)-hGRF (1-29)-NH_2, and met-enkephalin were from Bachem. Oxymetazoline, isoproterenol, and carbachol were from Sigma.

RESULTS

GRF binding to the human GRF receptor expressed in yeast. High level functional expression of the human GRF receptor in yeast was a necessary prerequisite to the development of a useful bioassay. The GRF receptor cDNA was placed under the control of the *GAL1* promoter in plasmid pJH25. These constructs also confer inducible overexpression of Gal4p, the transcriptional activating protein for galactose-inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane fractions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). The GRF receptor sequences were introduced into pJH25 without modification of the protein coding sequences. Previously, King *et al.* reported that replacement of the amino-terminal domain of the b_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast (9). In contrast, functional expression of GRF receptor in yeast does not require addition of any yeast sequences to the amino-terminus. A chimeric Gα protein composed of the proposed

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amino-terminal βγ-interaction domain from Gpalp and a carboxy-terminal receptor interaction domain from rat G_{as} (pLP122) under the control of the *GPA1* promoter was constructed. Yeast strains that contain expressed GRF receptor and chimeric G_a protein were assembled by transformation of a yeast strain (LY296) modified by deletion of genes encoding components of the mating signal transduction pathway with human GRF receptor (pJH25) and chimeric Gpa1-Gα_s protein expression (pLP122) plasmids.

The human GRF receptor retained agonist selectivity when expressed in yeast. A selective and sensitive bioassay was designed using a yeast strain (CY990) bearing the above described genetic modifications and plasmids conferring expression of the human GRF receptor (pJH25) and a Gpa1-Gα_s chimeric protein (pLP122). A dose-dependent growth response of CY990 cells was evident around an agonist analog of GRF [hGRF (1-29)-NH_2, Fig.25A] which was inhibited by coadministration of an antagonist analog [(D-arg^2)-hGRF (1-29)-NH_2, Fig. 25B]. The assay was selective: detectable growth responses were not observed in response to a variety of agonists selective for other G protein-coupled receptors (met-enkephalin, oxymetazoline, isoproteranol, carbachol), nor by yeast cells lacking the GRF receptor (data not shown). A detectable response was observed to 20 nmol of GRF, illustrating the sensitivity of the bioassay.

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adaptation pathway. J. Biol. Chem. **268**: 8070-8077.

EXAMPLE 13

Overexpression of *STE50* enhances the sensitivity of yeast bioassay.

Several molecular mechanisms for adaptation have been described for the yeast mating signal transduction pathway (1). Alterations to one or more of these mechanisms should serve to enhance the sensitivity of a bioassay by altering desensitization pathways and, therefore, prolonging the signal initiated by agonist binding to receptor. The effects of an *sst2* mutation on the sensitivity of the yeast bioassay were described previously (Example 6). As an alternative to the genetic modification at *sst2*, overexpression of the yeast *STE50* gene was predicted to have similar effects (2), although by a different mechanism of action (2, 3). The *STE50* gene was isolated and placed under the control of a strong constitutive promoter in a high-copy-number plasmid resulting in significant overexpression of the gene. Yeast engineered to respond to the mammalian hormone somatostatin through an expressed SSTR2 somatostatin receptor were found to exhibit a more robust response to hormone if *STE50* was overexpressed.

MATERIALS AND METHODS

Construction of *STE50* expression plasmid. Growth of bacterial strains and plasmid manipulations were performed by standard methods (4). The protein coding sequences for *STE50* were amplified by polymerase chain reaction (PCR) using oligonucleotides selected by examination of the published sequence (2). The sense

oligonucleotide (5'- GTCGACAAATCAG ATG GAG GAC GGT AAA CAG G -3') contained the translation start codon (underlined) and a *Sal*I restriction site and the antisense oligonucleotide (5'- GAGCTCA TTA GAG TCT TCC ACC GGG GG -3') contained the translation stop codon (underlined) and a *Sac*I restriction site. These oligonucleotides were used as primers in a standard PCR to amplify *STE50* from *Saccharomyces cerevisiae* genomic DNA. The 1,100 basepair amplification product was cloned into the pCR2 vector (Invitrogen Corp., San Diego, CA) and confirmed by DNA sequencing. The *STE50* sequences were then isolated on a *Sal*I-*Sac*I restriction fragment and cloned into a pADH expression vector (5), placing the expression of *STE50* under the control of the strong constitutive *ADH1* promoter. This plasmid was designated pOZ162.

Yeast strain construction. Growth and transformation of yeast strains were performed as described by Rose *et al.* (6). The SSTR2 somatostatin receptor expression strain LY268 (*MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS3 sst2ΔADE2*, pJH2, pLP82) was described in prior examples and by Price *et al.* (7). Strain LY268 was transformed with either the *STE50* expression plasmid pOZ162 or the pADH vector. These strains are denoted CY560 or CY562, respectively.

Bioassay. Bioassay of SSTR2 somatostatin receptor expressed in yeast was described in prior examples and by Price *et al.* (7). Briefly, yeast strains were grown overnight in 2 ml synthetic complete liquid medium containing glucose (2%) and lacking uracil, tryptophan and leucine (SCD-ura-trp-leu), washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2%)-ura-trp-leu liquid medium. Molten (52° C) SC Galactose (2 %)-ura-trp-leu-his agar medium (30 ml, adjusted to pH 6.8 by addition of KOH prior to autoclaving) was inoculated with 0.06 ml of the overnight

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culture to produce a final cell density of approximately 10^5 cells/ml and poured in square (9 x 9 cm) petri plates. Sterile filter discs were placed on the surface of the solidified agar and saturated with 10 µl of sterile water containing the indicated amounts of somatostatin-14 (Bachem Bioscience Inc., Philadelphia, PA) or α mating pheromone (Sigma, St. Louis, MO). Plates were incubated at 30° C for 3 days.

RESULTS

The effect of *STE50* overexpression on the sensitivity of the yeast bioassay was examined by comparing strains differing only in the level of *STE50* expression (Fig. 26). Bioassay plates were made containing either the *STE50* overexpression strain CY560 or the control strain CY562. The responses of these strains to somatostatin or yeast pheromone were examined as described in Materials and Methods. Both strains had very weak responses to yeast pheromone because the wild-type yeast Gα gene, *GPA1*, had been disrupted and functionally replaced by a gene expressing a chimeric Gpa1/$G\alpha_{i2}$ protein (7). This chimeric Gα protein does not efficiently interact with the yeast pheromone receptor. However, the response of these cells to somatostatin is strong (Fig. 26). As predicted, the overexpression of *STE50* resulted in a more robust response (Fig. 26a). These data demonstrate that the overexpression of *STE50* produces a hypersensitivity to ligands acting through G protein-coupled receptors coupled to the yeast signal transduction pathway, even if the ligand and receptor originate from a heterologous source.

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EXAMPLE 14

IDENTIFICATION OF COMPOUNDS WITH SOMATOSTATIN RECEPTOR AGONIST AND/OR ANTAGONIST PROPERTIES

Novel subtype-selective compounds with somatostatin agonist properties have significant therapeutic potential in the detection and treatment of various types of cancer. Compounds with somatostatin antagonist properties may be useful in promoting growth hormone release in agricultural species. Increased growth hormone release may lead to useful improvements in growth performance and carcass quality. To these ends, a yeast-based mechanism-based screening assay was developed to assay compounds for those that possessed desirable somatostatin agonist and/or antagonist properties.

Bioassay. A bioasssay designed to detect compounds with somatostatin agonist and/or antagonist properties was mobilized using a yeast strain (LY364 MAT**a** ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUS1-HIS33 sst2ΔADE2, pJH2, pLP82) that functionally expressed the rate SSTR2. The assay was accomplished using a modicication of a standard procedure. Y364 was grown overnight in 2 ml synthetic complete liquid medium containing glucose (2 %) and lacking uracil and typtophan (SCD-ura-trp) medium, washed to remove residual glucose, and grown overnight in 5 ml SC Galactose (2 %)-ura-trp liquid medium. Molten (55°C) SC Galactose (2 %)-ura-trp-his agar medium (150 ml, adjusted to pH 6.8 by addition of concentrated (2 x 10^4 cells/ml) and plated in square (500 cm^2) petri plates For assay of antagonists, somatostatin (20 nM S-14) was added to the molten agar prior to pouring. Sterile filter disks were placed on the surface of the solidified agar and saturated with 10µl of sterile water containing candidate compounds. Plates were incubated at 30° C for 3 days.

Results. Active compounds from a primary screen were reassayed and the results displayed in Figure 27. The

left hand panel displays the results of an assay for compounds with somatostatin agonist properties. Four compounds exhibited substantial growth promoting activity expected of compounds with somatostatin agonist properties. The compounds found in the bottom left four positions are varying amounts of somatostatin applied as controls. The right hand panel displays the results of an assay for compounds with somatostatin antagonist activity. In the antagonist bioassay, somatostatin is added to the molten agar prior to pouring. In this way, all cells within the plate are induced to grow in response to somatostatin. As applied active compounds with antagonist properties diffuse into the agar medium and come into contact with the cells within, the growth response induced by somatostatin is interrupted, yielding a clear zone of inhibited growth. Several compounds exhibited detectable growth inhibiting properties.

EXAMPLE 15

Fusion of *STE2* sequences to the amino terminal of SSTR2 reduces signaling efficiency in response to somatostatin.

High level functional expression in yeast of G protein-coupled receptors in general, and the SSTR2 in particular, was a necessary prerequisite to the development of a useful biassay. King *et al.* reported that replacement of the amino-terminal domain of the β_2-adrenergic receptor with equivalent *STE2* sequence was necessary for efficient receptor expression in yeast. To test this hypothesis and the effect of *STE2* sequences on expression of the somatostatin receptor in yeast, the rat SSTR2 cDNA was placed under the control of the *GAL1* promoter in plasmids pJH1 and pJH2. These constructs confer inducible overexpression of Gal4p, the transcriptional activating protein for galactose-

inducible genes, resulting in significantly elevated levels of receptor protein in crude membrane franctions compared to receptor expressed from a plasmid lacking *GAL4* sequences (data not shown). In SSTR2 expression plasmid pJH1, DNA sequences encoding the first 13 amino acids of SSTR2 were replaced with coding sequence for the first 23 amino acids of STE2 (Fig. 11). The rate SSTR2 sequences were introduced into pJH2 without modification of the protein coding sequences. Yeast strains containing these constructs (LY322: *MATa ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUSI-HIS3 sst2ΔADE2, pJH1, pLP82; LY268: MATa ura3-52 trp1Δ63 his3Δ200 ade2-101 lys2-801 gpa1ΔhisG far1ΔLYS2 FUSI-HIS3 sst2ΔADE2, pJH2, pLP82)* bear a plasmid (pLp82) that confers expression of a chimeric Gα protein composed of the proposed amino-terminal βγ-interaction domain from Gpalp and carboxy-terminal receptor interaction domain from rat $G_{\alpha i2}$ under the control of the GPA1 promoter. The magnitude of the response of these strains to applied somatostatin (S-14) was measured (Fig. 28). LY268 cells exhibited a robust growth response to applied S-14, demonstrating that rat SSTR2 does not require *STE2* sequences to be functionally expressed in yeast (Fig. 28A) The growth response of LY268 cells was substantially greater than that exhibited by LY322 cells (Fig. 28B). The sole difference between these strains is the presence of *STE2* sequences in pJH1 found in LY322. Thus, replacement of the amino terminum of SSTR2 with the equivalent segment of *STE2* greatly reduces the efficiency of signalling in response to applied somatostatin. In spite of the observations of King *et al.*, heterologous G protein-coupled receptors expressed in yeast do not require amino-terminal protein coding sequences from any yeast protein for functional expression.

What is claimed is:

1. A transformed yeast cell comprising a first nucleotide sequence which codes for a heterologous G protein coupled receptor and a second nucleotide sequence which codes for all or a portion of a G protein complex.

2. The transformed yeast cell of Claim 1, wherein said first nucleotide sequence is selected from the group consisting essentially of β2-adrenrgic receptor, α2-adrenergic receptor, 5HT-1A receptor, muscarinic acetylcholine receptor, growth hormone releasing factor receptor, cholecystokinin receptor, adenosine receptor, and somatostatin receptor.

3. The transformed yeast cell of Claim 1, wherein said second nucleotide sequence codes for a Gα protein selected from the group consisting essentially of yeast Gα, heterologous Gα, and chimeric Gα.

4. The transformed yeast cell of Claim 2, wherein said second nucleotide sequence codes for a Gα protein selected from the group consisting essentially of yeast Gα, heterologous Gα, and chimeric Gα.

5. The transformed yeast cell of any of Claims 1-4 further comprising a pheromone-responsive promoter and a reporter gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith.

6. The transformed yeast cell of any of Claims 1-4 further comprising a pheromone-responsive promoter and a reporter gene positioned downstream from the pheromone-responsive promoter and operatively associated therewith, wherein said reporter gene is

selected from the group consisting essentially of *HIS3*, G418r, *URA3*, *CYH2*, *LYS2*, *CAN1* and *LacZ* genes.

7. The transformed yeast cell of any of Claims 1-4 further comprising a reporter gene construct *FUS1-HIS3*.

8. The yeast cell of any of Claims 1-4 further comprising at least one gene mutation selected from the group consisting essentially of *fus3*, *far1*, *sst2*, *sgv1*, *ste2*, *ste3*, *pik1*, *msg5*, *sig1*, *afr1*, and STE50.

9. The yeast cell of Claim 8 wherein said mutation is a deletion.

10. The yeast cell of Claim 8 wherein said mutation is an overexpression.

11. The yeast cell of Claim 10 wherein said gene is *STE50*.

12. The yeast cell of Claim 3 further comprising a mutation at a *FAR1* gene in conjunction with at least one mutation at a gene selected from the group consisting essentially of *SST2*, *STE50*, *SGV1*, *STE2*, *STE3*, *PIK1*, *MSG5*, *SIG1*, and *AFR1*.

13. The yeast cell of Claim 12 further comprising a reporter gene construct.

14. The yeast cell of Claim 13 wherein said reporter gene is *FUS1-HIS3*.

15. The yeast cell of Claim 3 further comprising a mutation in a *SCG1/GPA1* gene.

BNSDOCID: <WO 9521925A1 I >

16. The yeast cell of Claims 3 or 13 wherein said heterologous Gα subunit comprises a subunit selected from the group consisting essentially of a Gs subunit, Gi subunit, Go subunit, Gz subunit, Gq, G11, and G16.

17. The yeast cell of Claims 3 or 13 wherein said Gα subunit is a chimeric subunit.

18. The yeast cell of Claims 3 or 13 wherein said Gα subunit is a chimeric subunit comprising the amino terminal domain of yeast *GPA1/SCG1* and the carboxy terminal domain of a heterologous $G\alpha_{i2}$.

19. The yeast cell of Claims 3 or 13 wherein said Gα subunit is $G\alpha_{i2}$ and a Gβγ complex is an endogenous yeast Gβγ complex.

20. The yeast cell of Claim 1 further comprising at least a portion of a nucleotide sequence encoding for a yeast G protein coupled receptor positioned upstream to said nucleotide sequence encoding for said heterologous G protein receptor and operatively associated therewith, to encode for a chimeric G protein coupled receptor.

21. The chimeric receptor of Claim 20 wherein said nucleotide sequence encoding for all or a portion of a yeast G protein coupled receptor comprises a yeast gene selected from the group consisting essentially of the group consisting of *STE2* and *STE3*.

22. The yeast cell of Claim 21 further comprising a nucleotide sequence encoding a chimeric G protein comprising yeast nucleotide sequences and sequences froma heterologous G protein.

23. The yeast cell of any of Claims 3, 14, and 20 further comprising a heterologous Gβγ complex.

24. A chimeric G protein coupled receptor encoded by the nucleotide sequence of Claim 20.

25. A nucleotide expression vector capable of expressing a heterologous G protein-coupled receptor into a cell membrane of a yeast cell.

26. A nucleotide expression vector of Claim 25 further capable of expressing Gα, β, and γ proteins into a yeast cell.

27. A method of assaying compounds to determine effects of ligand binding to a heterologous G protein coupled receptor expressed on the surface of yeast cells comprising the steps of:

a) providing a culture of the transformed yeast cell of Claim 1;

b) contacting a selected compound to said cells to allow binding of said compound to the heterologous G protein receptor expressed on the cell surface; and

c) determining the growth of said cells as an indication of the activation of the heterologous G protein coupled receptor upon binding of said compound.

28. The method of assaying compounds of Claim 27 to determine effects on cell growth comprising:

(a) providing cultures of the yeast cell of Claim 1 by plating the yeast cells in agar medium

comprising a cognate agonist for a heterologously expressed receptor;

(b) applying selected compounds to the agar plate; and

(c) identifying compounds that inhibit agonist dependent activation of the heterologous receptor.

29. The transformed yeast cell of any of Claims 1-4 further comprising a reporter system selected from the group consisting essentially of heterologous adenylylcyclase, heterologous G protein-coupled potassium channel, and heterologous PLC-β.

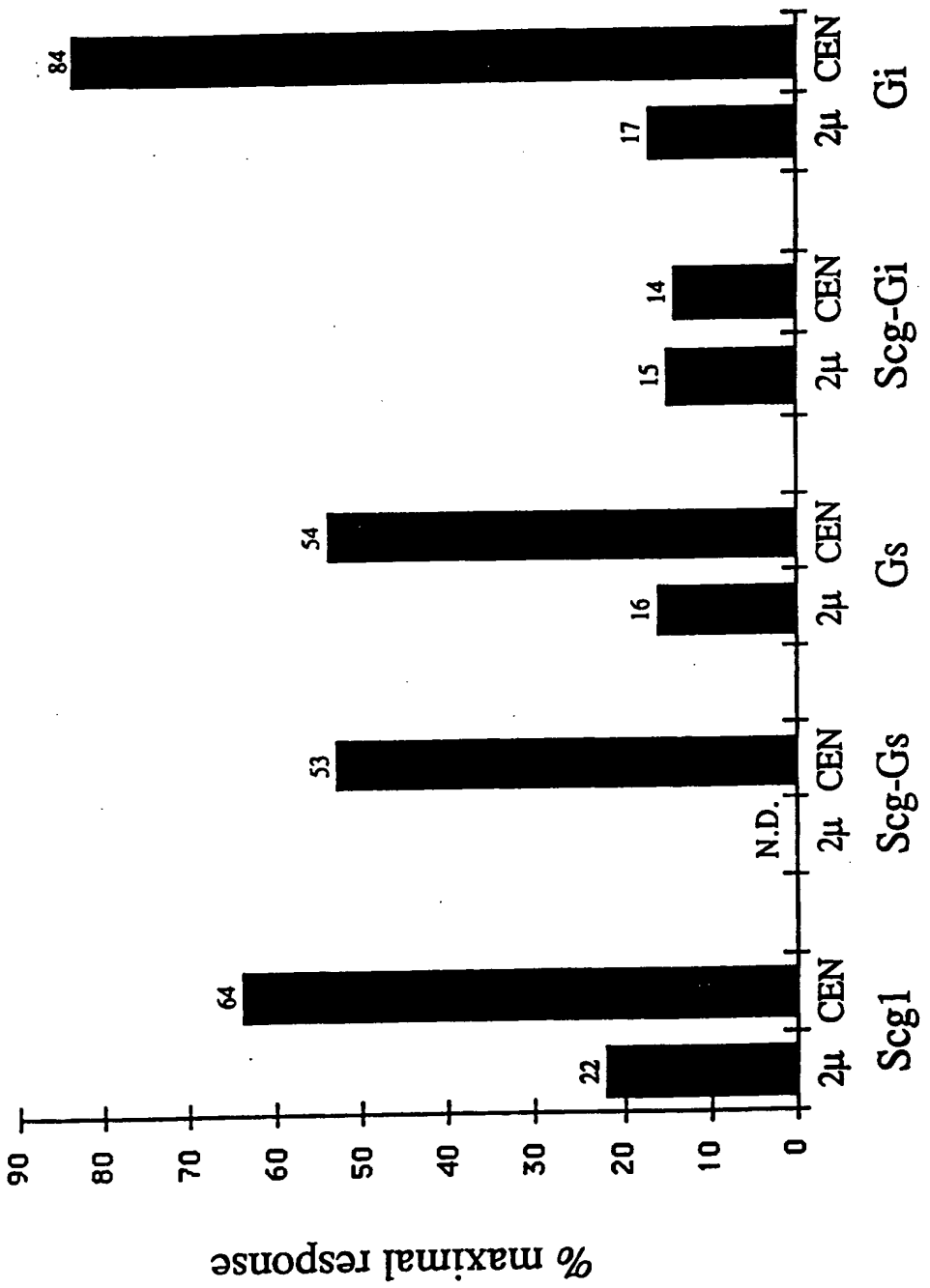

% maximal response
0
10
20
30
40
50
60
70
80
90
22
64
N.D.
53
16
54
15
14
17
84
2μ
CEN
Scg1
Scg-Gs
Gs
Scg-Gi
Gi

FIG. 1

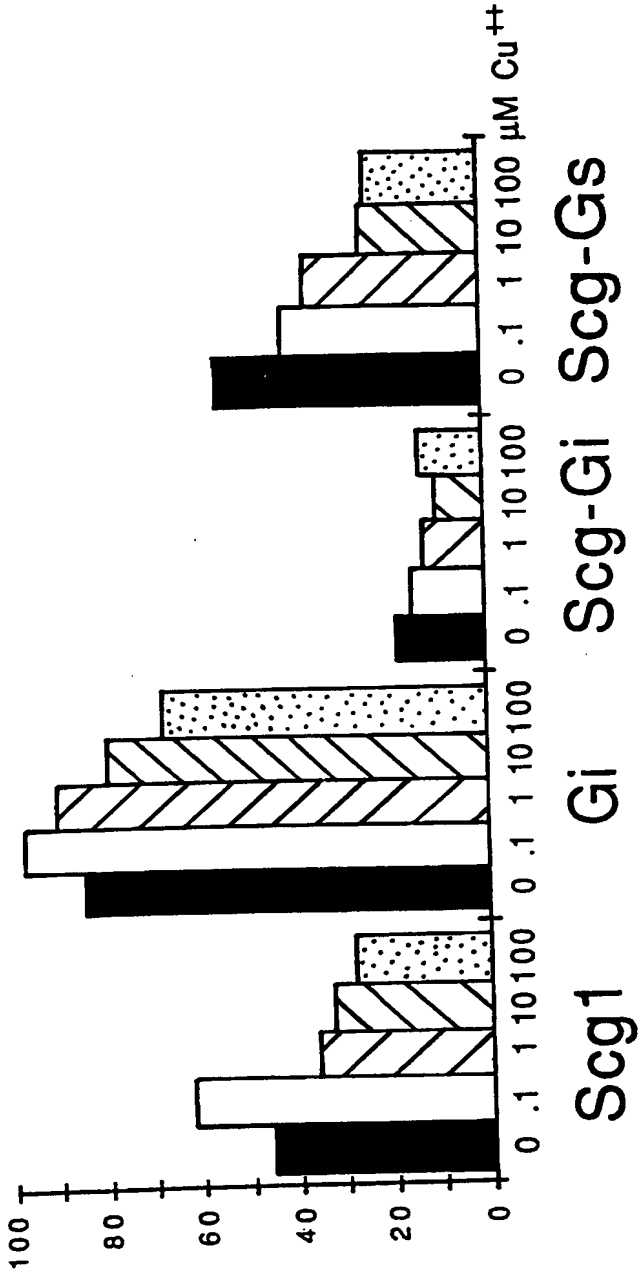

100
80
60
40
20
0
0 .1 1 10 100
Scg1
0 .1 1 10 100
Gi
0 .1 1 10 100
Scg-Gi
0 .1 1 10 100 µM Cu++
Scg-Gs

FIG. 2

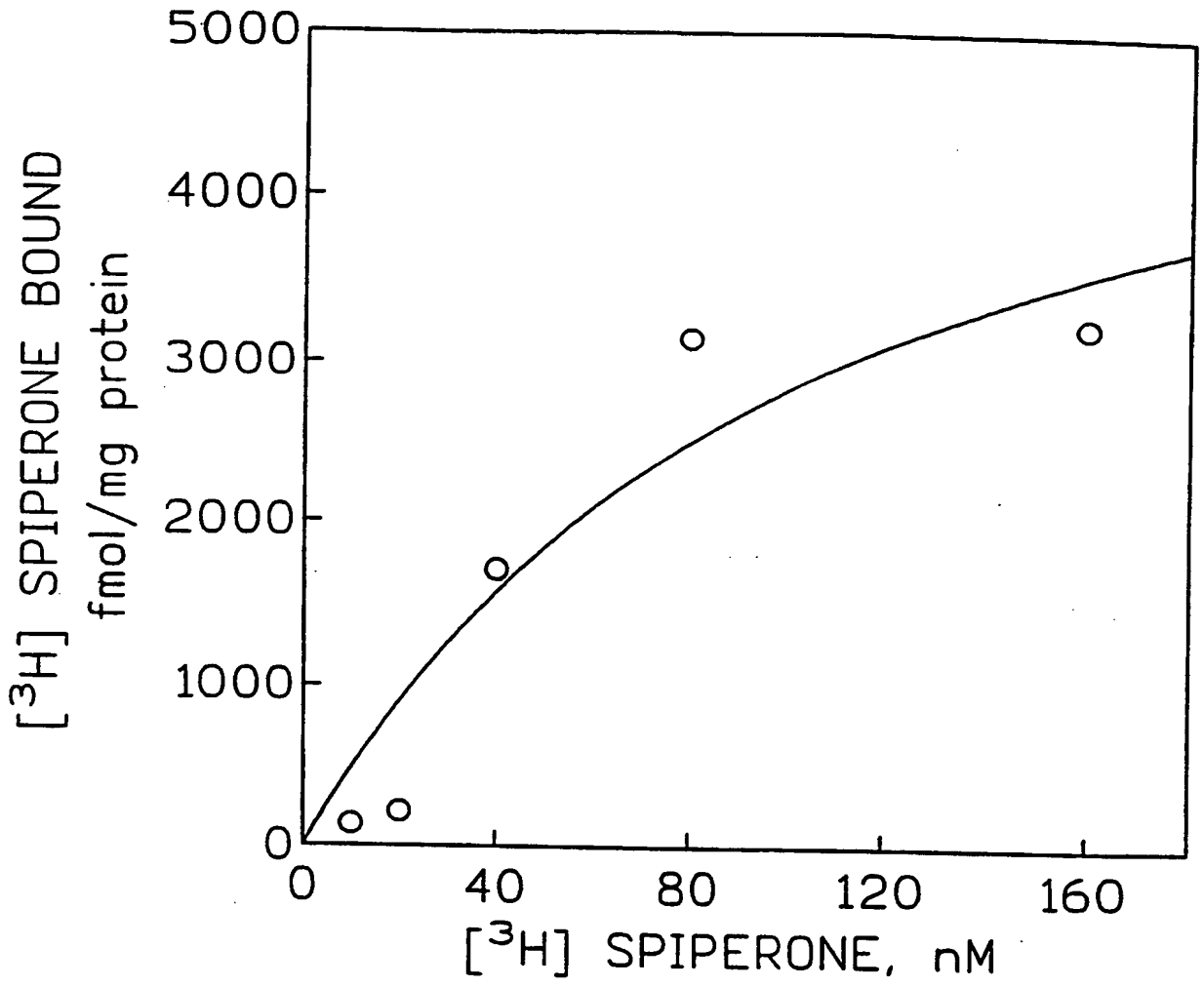

5000
4000
3000
2000
1000
0
[3H] SPIPERONE BOUND
fmol/mg protein
0
40
80
120
160
[3H] SPIPERONE, nM

FIG. 3

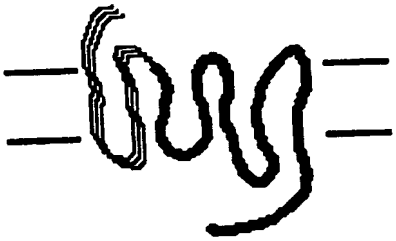
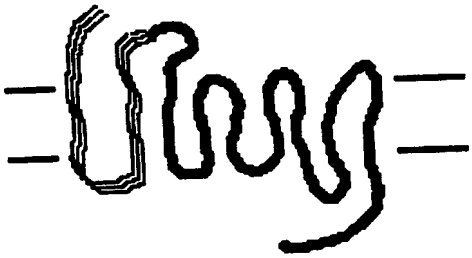
		Bmax (pmol/mg)
CHI11		3.1
CHI17		1.6
CHI18		0.7

FIG. 4

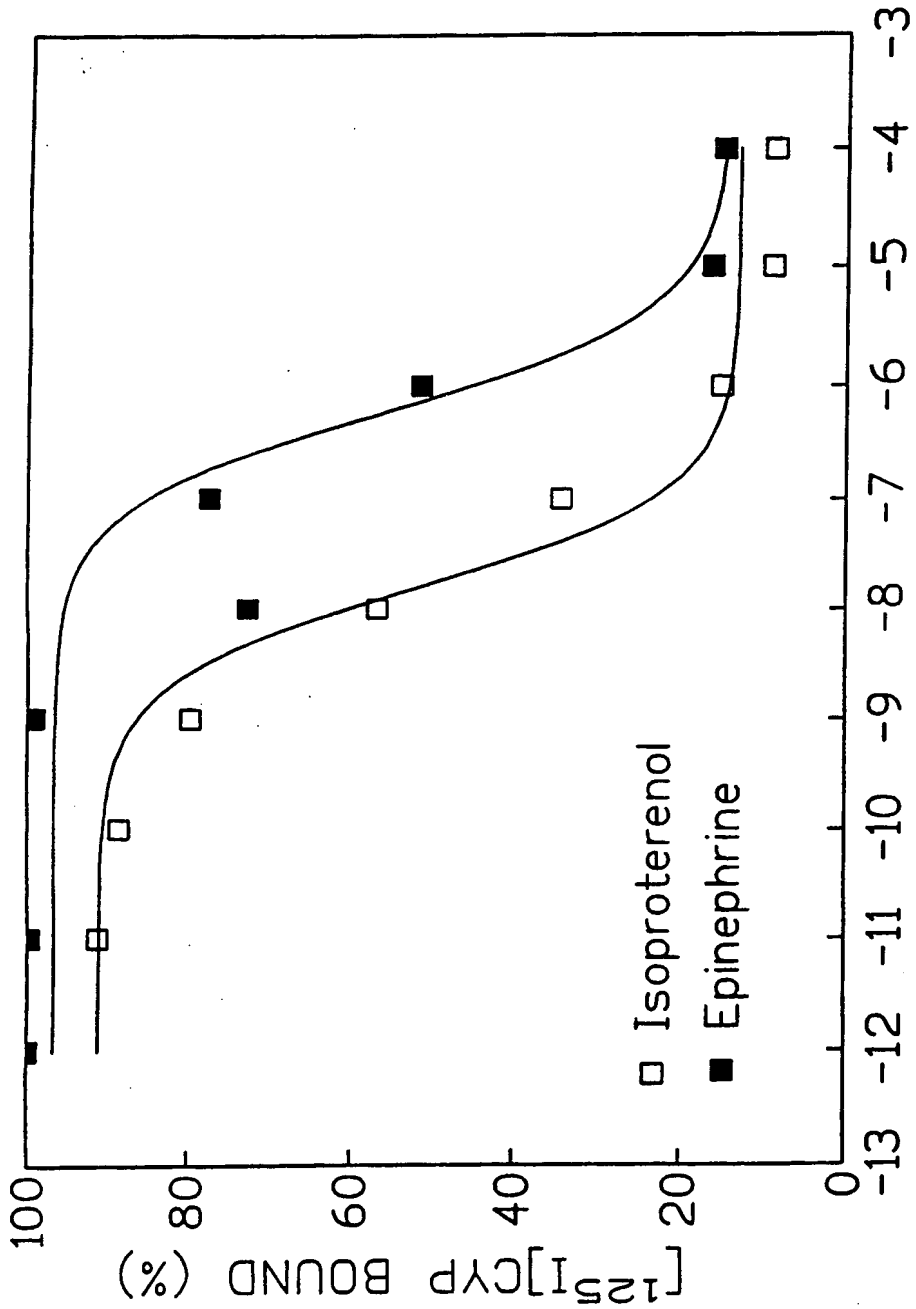

[125I]CYP BOUND (%)
100
80
60
40
20
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3
LOG10 [COMPETITOR], M
Isoproterenol
Epinephrine

FIG. 5

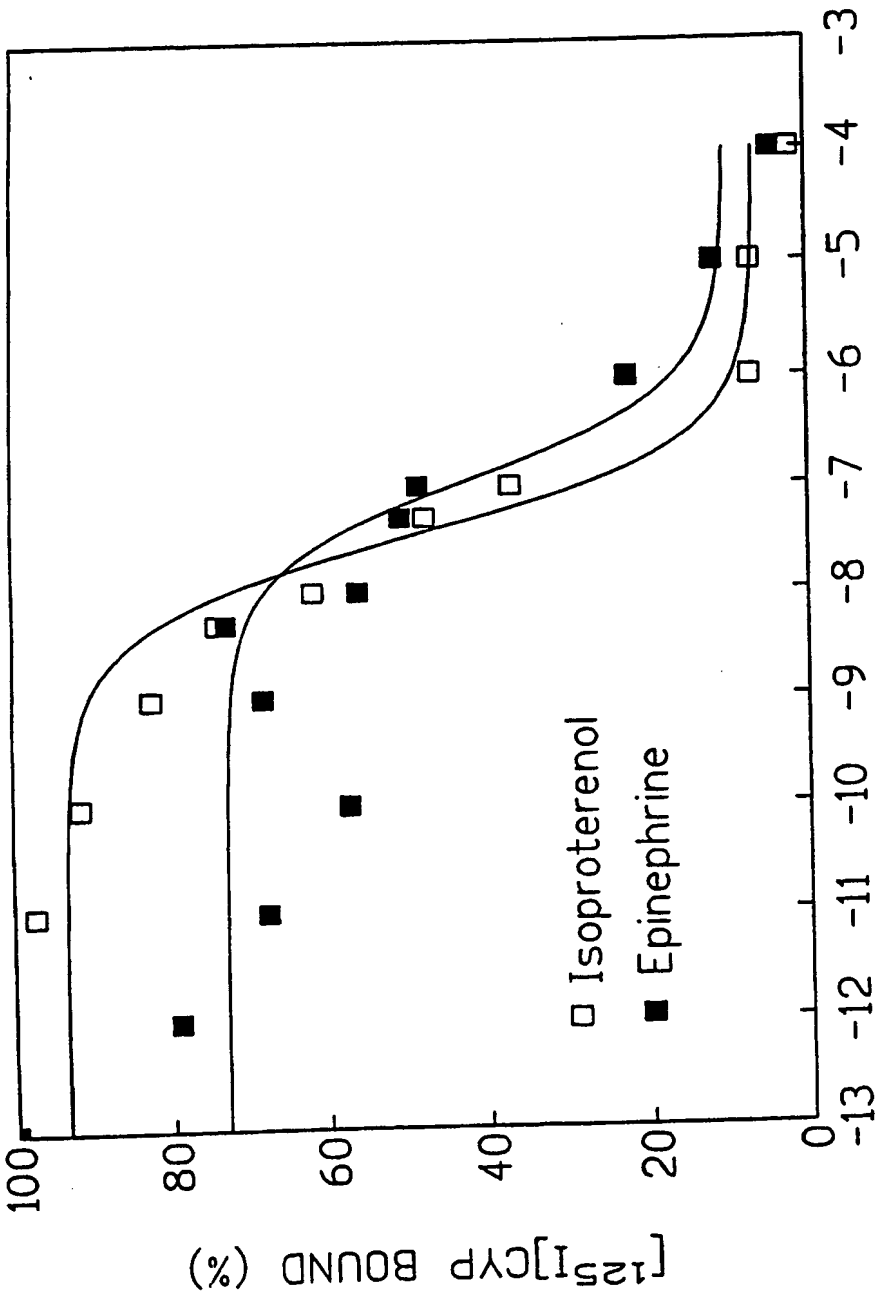

[125I]CYP BOUND (%)
100
80
60
40
20
0
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
LOG10 [COMPETITOR], M
Isoproterenol
Epinephrine

FIG. 6

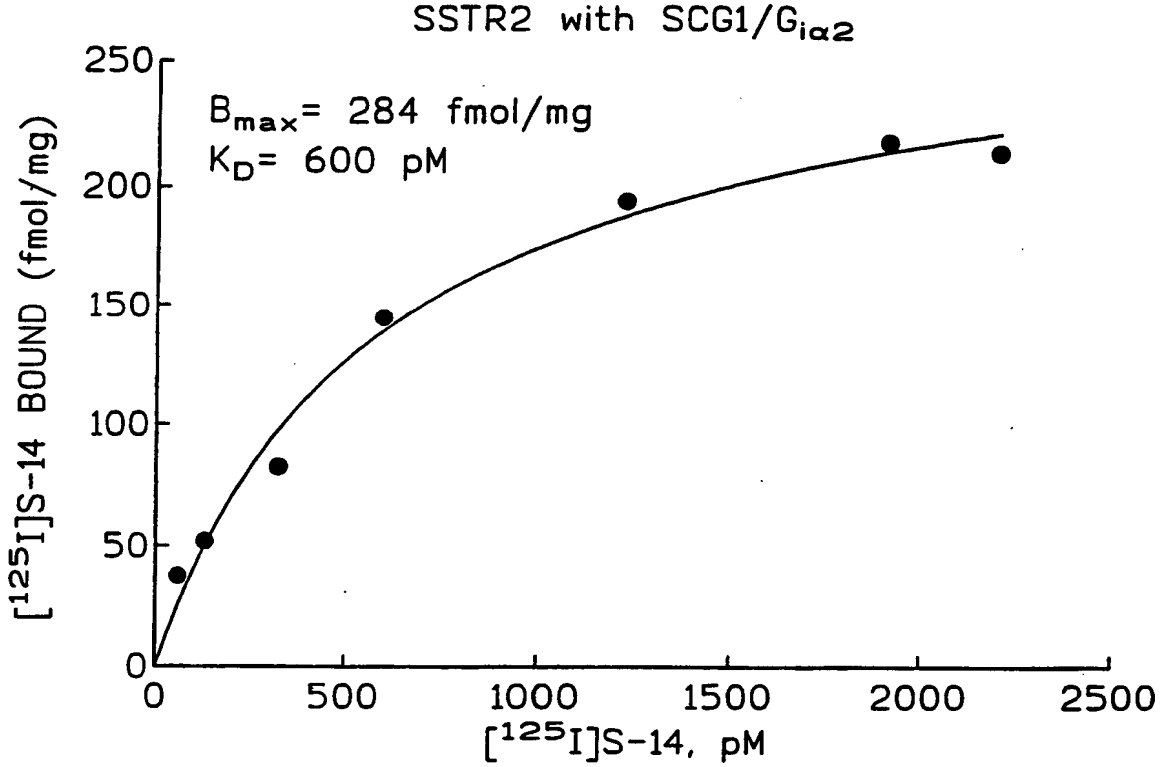

SSTR2 with SCG1/$G_{i\alpha 2}$
B_{max}= 284 fmol/mg
K_D= 600 pM
[^{125}I]S-14 BOUND (fmol/mg)
250
200
150
100
50
0
0
500
1000
1500
2000
2500
[^{125}I]S-14, pM

FIG. 7

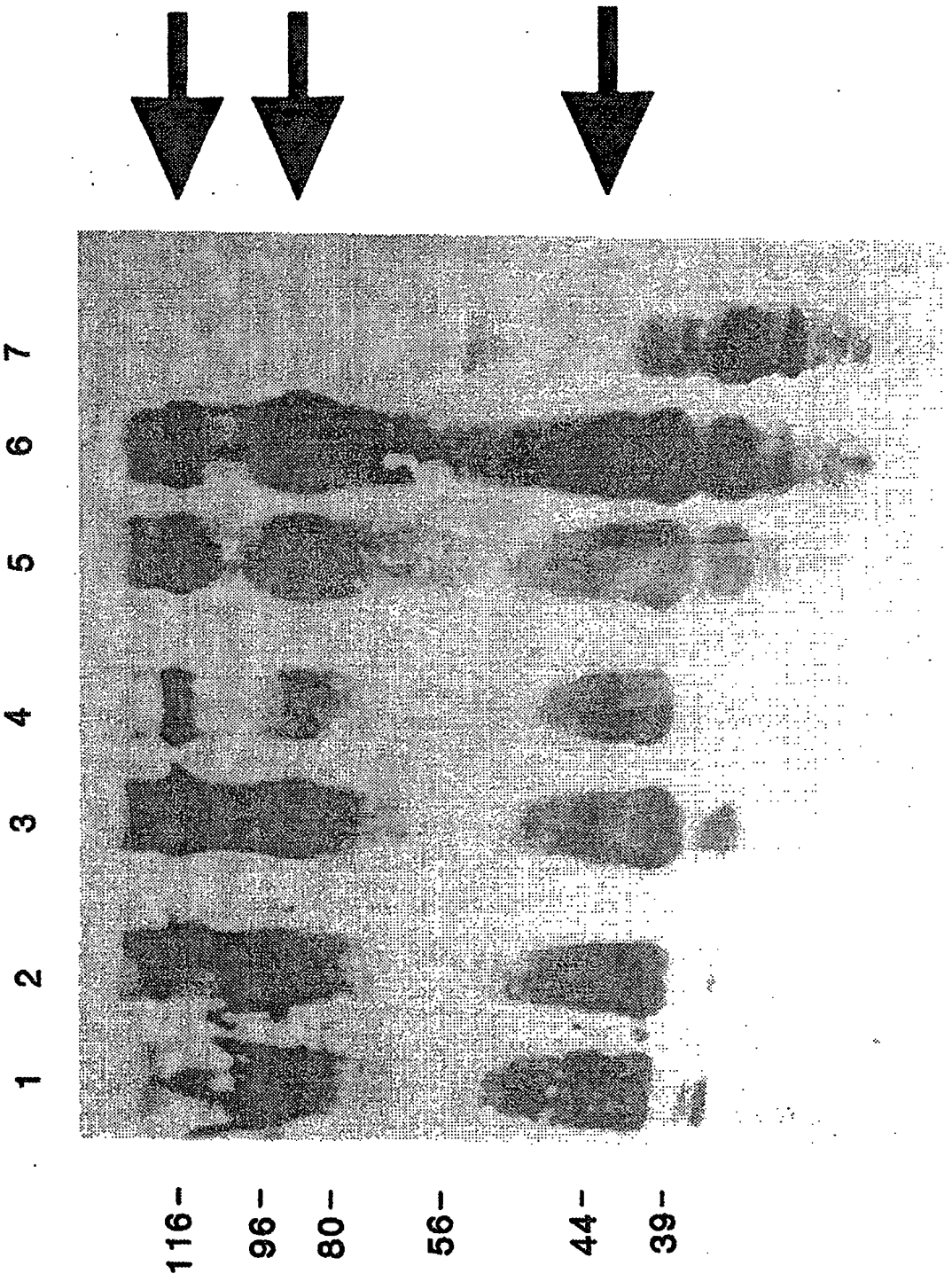


FIG. 8

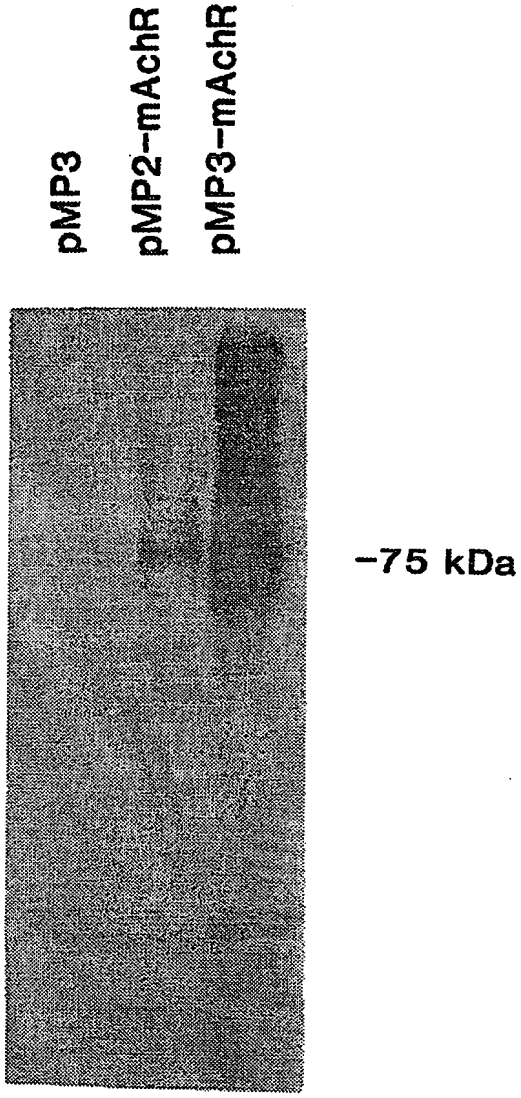


FIG. 9

FIG. 10

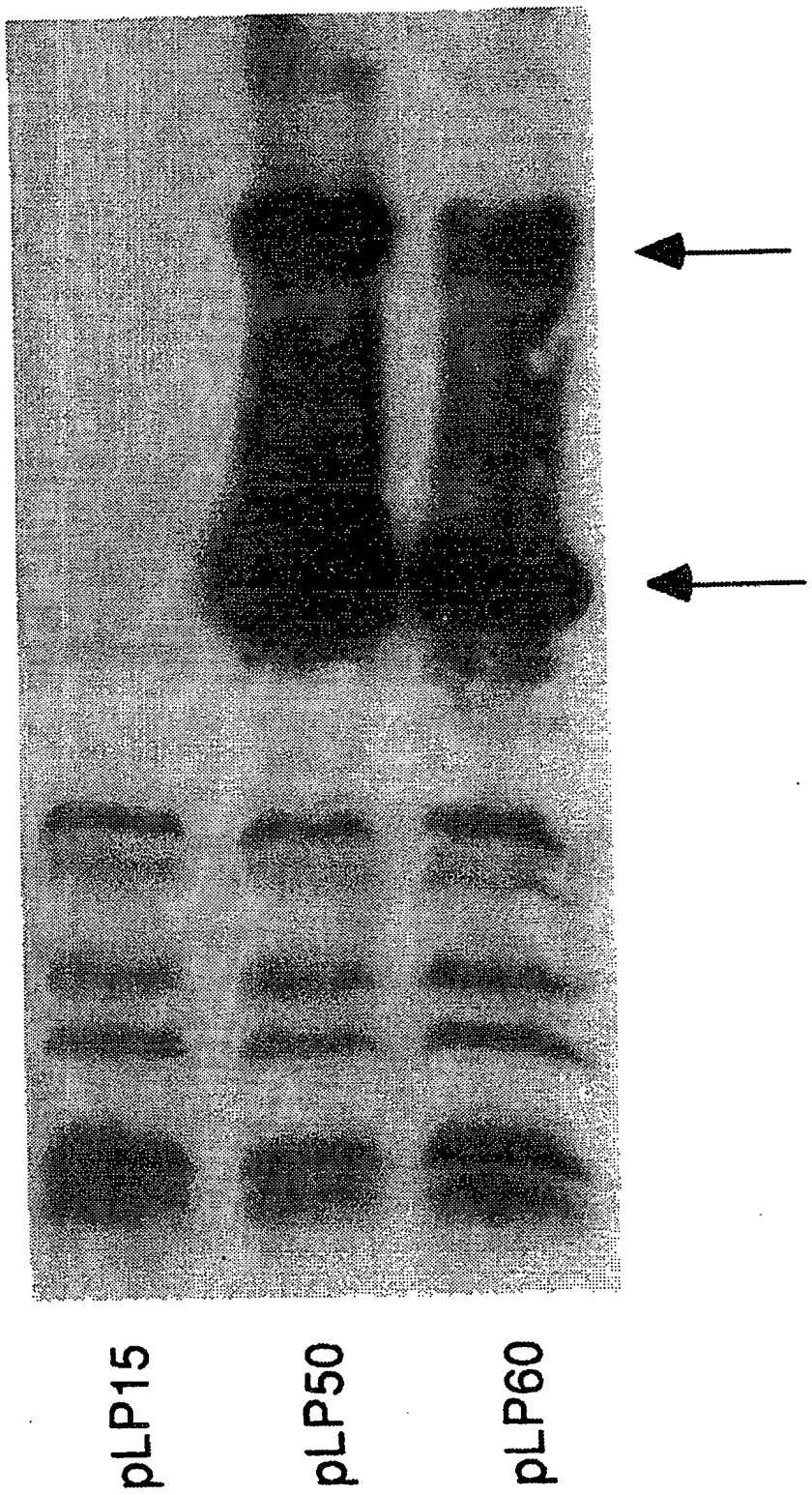
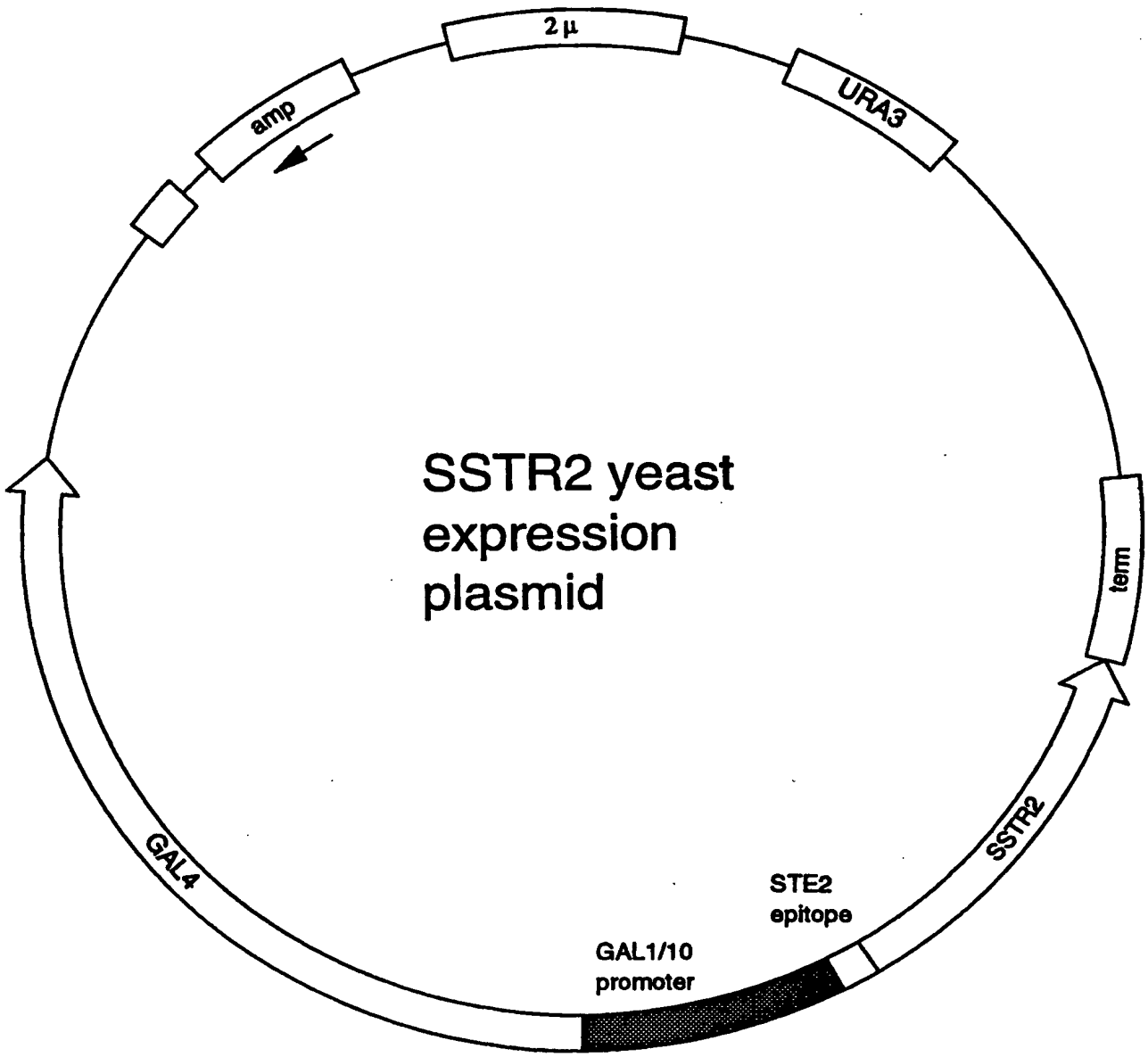


FIG. 11


2μ
URA3
amp
term
SSTR2
STE2
epitope
GAL1/10
promoter
GAL4
SSTR2 yeast
expression
plasmid

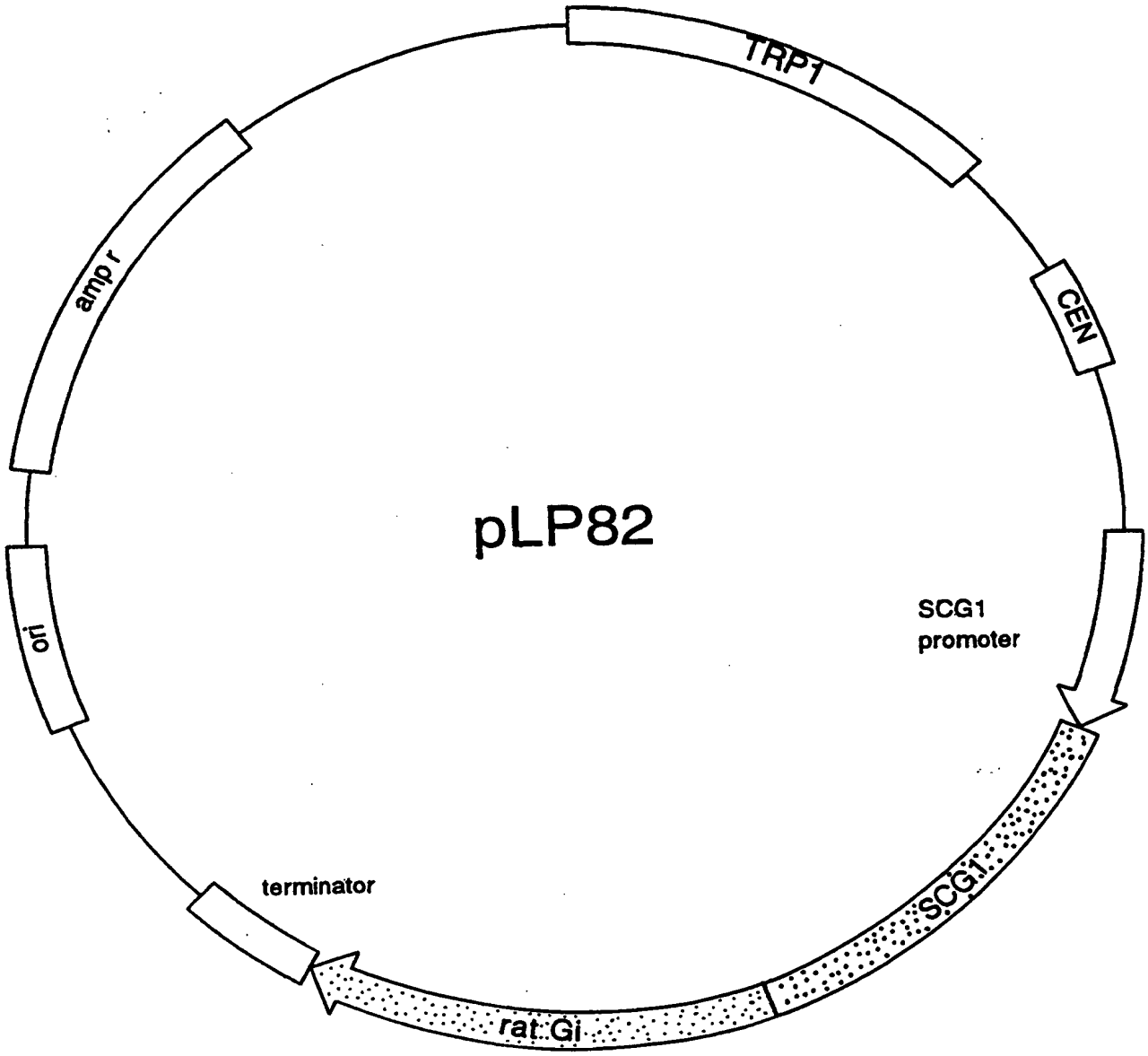

TRP1
CEN
SCG1
promoter
SCG1
rat Gi
terminator
ori
amp r
pLP82

FIG. 12

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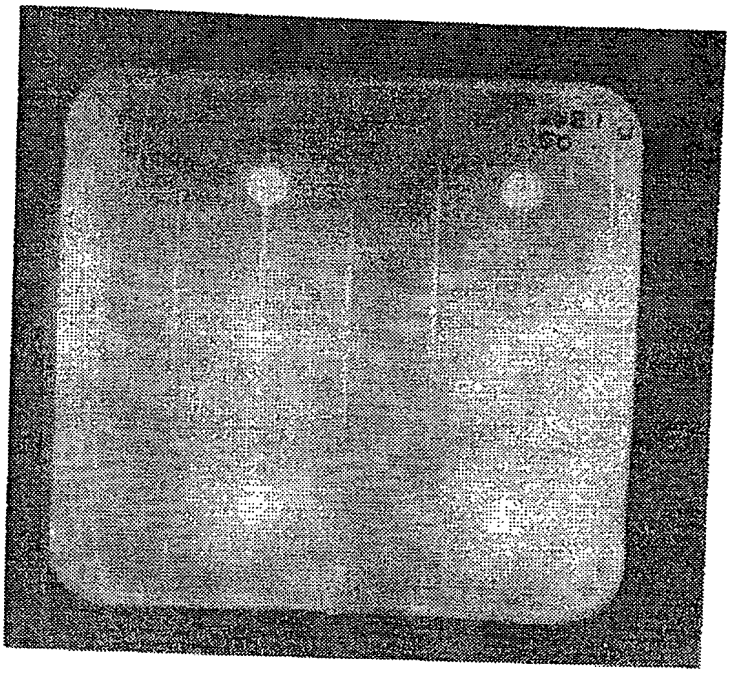
CEN pSCG1-Scg-Gαi2



17 nmol enk

100 nmol MK678

10 nmol MK678

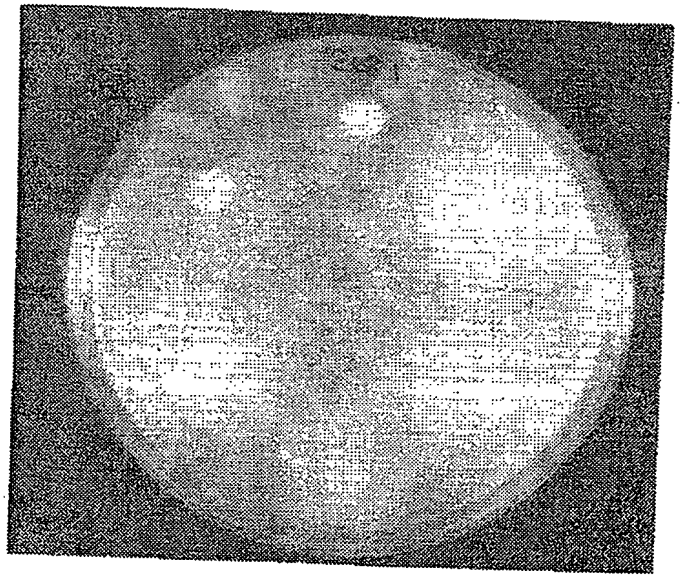
blank

60 nmol S14

6 nmol S14

FIG. 13A

blank



17 nmol enk

60 nmol S14

10 nmol MK678

100 nmol MK678

6 nmol S14

FIG. 13B

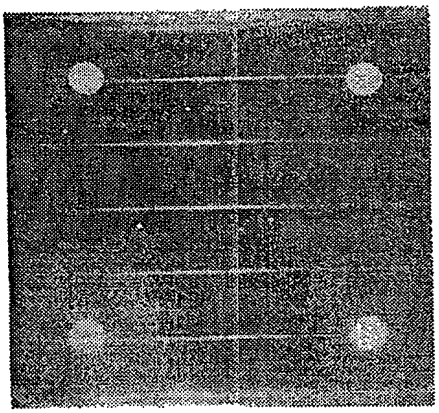


FIG. 14B

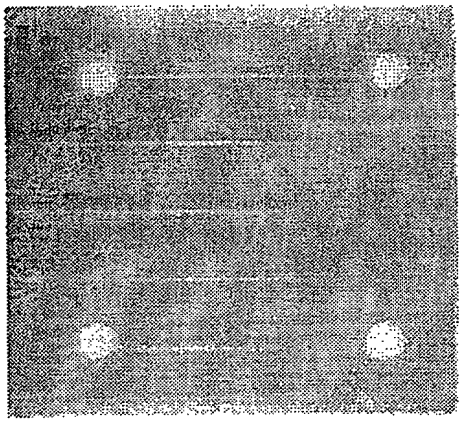


FIG. 14D

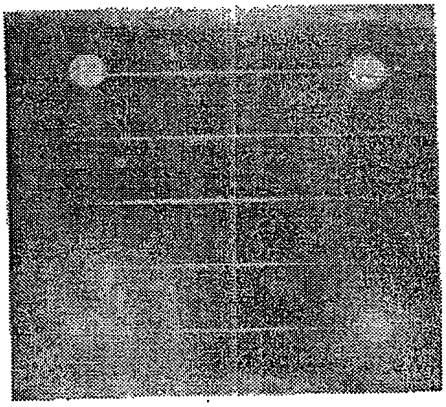


FIG. 14A

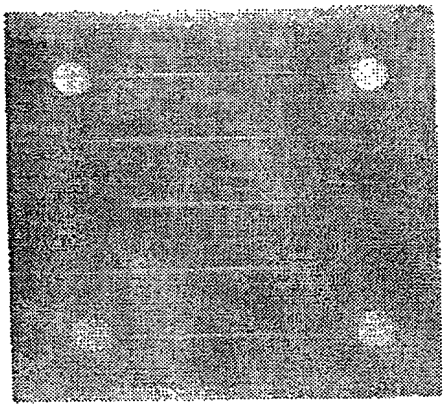


FIG. 14C

SUBSTITUTE SHEET (RULE 26)

CEN pSCG1-Scg1

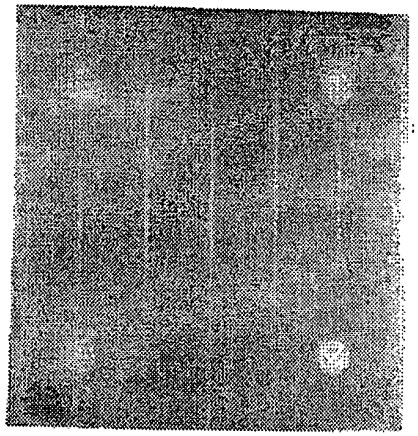


FIG. 15A

CEN pPGK-Scg1

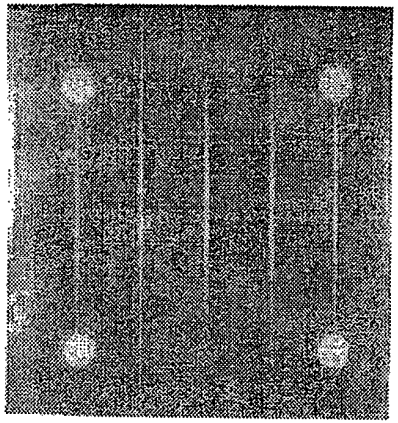
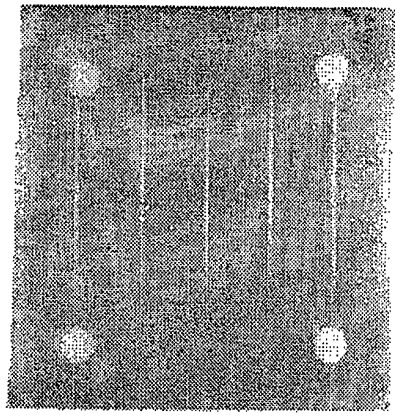
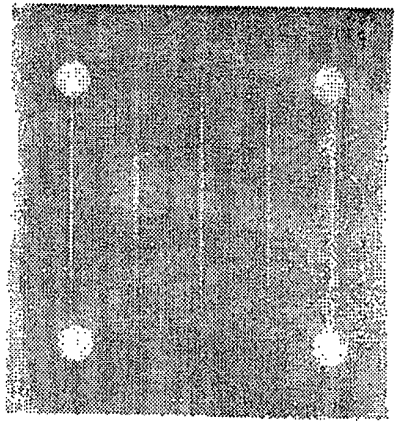


FIG. 15B



2 μ pSCG1-Scg1

FIG. 15C



2μ pPGK-Scg1

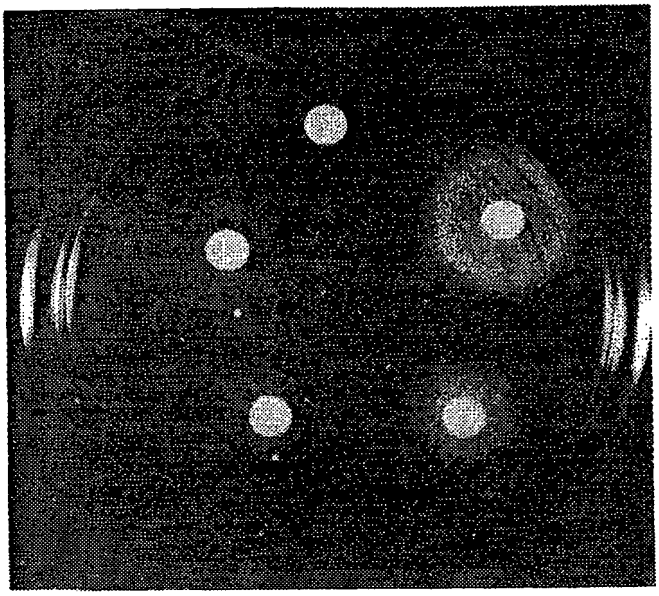
FIG. 15D

16/28

Carrier

0.001 nmol

1 nmol



0.01 nmol

0.1 nmol

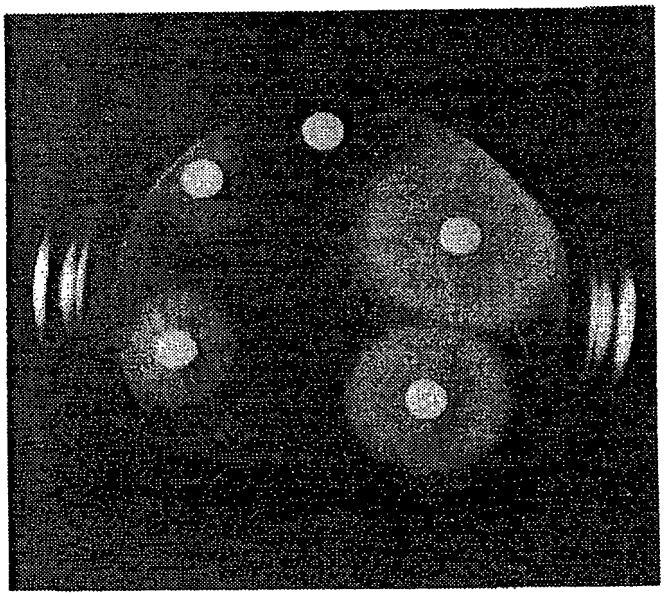
LY230 (SST2)

FIG. 16A

Carrier

0.001 nmol

1 nmol



0.01 nmol

0.1 nmol

LY238 (sst2)

FIG. 16B

SUBSTITUTE SHEET (RULE 26)

CEN pSCG1-Scg1
sst2Δ

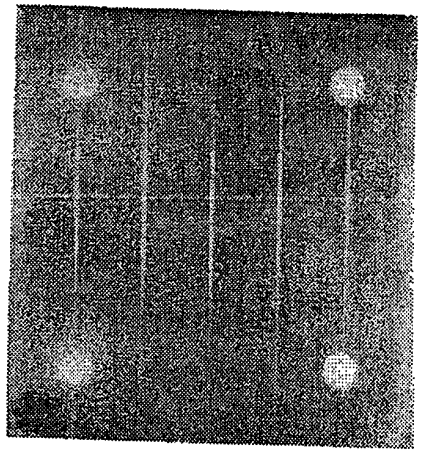


FIG. 17A

CEN pSCG1-Scg-Gαi2
sst2Δ

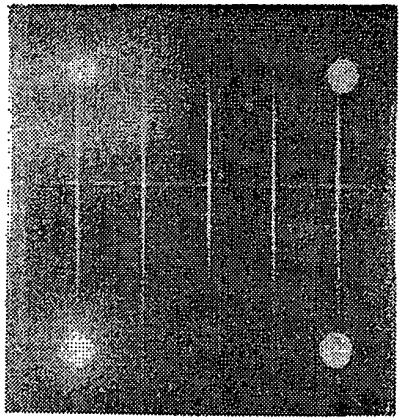
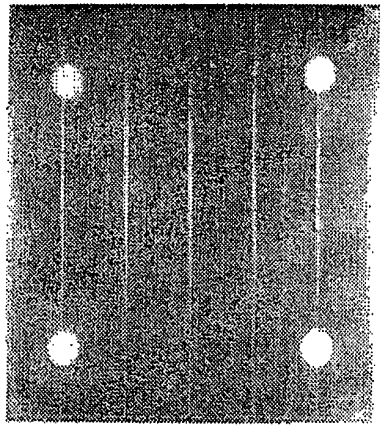
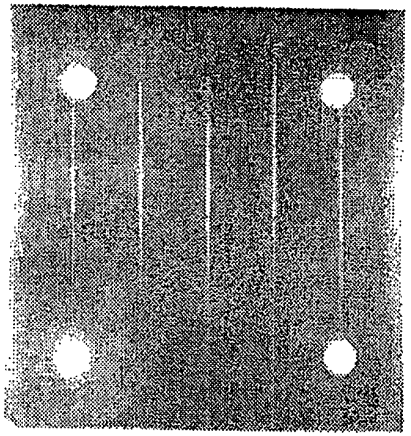


FIG. 17B



CEN pSCG1-Scg1
SST2

FIG. 17C



CEN pSCG1-Scg-Gαi2
SST2

FIG. 17D

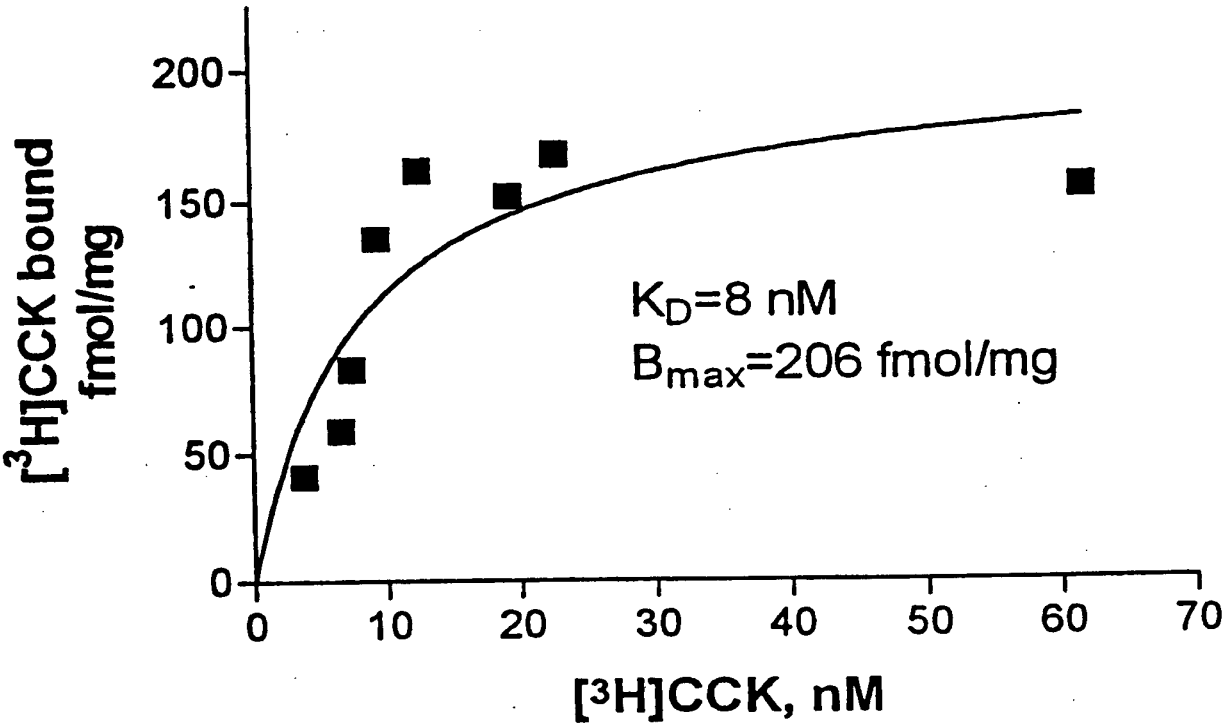

[3H]CCK bound fmol/mg
200
150
100
50
0
0
10
20
30
40
50
60
70
[3H]CCK, nM
KD=8 nM
Bmax=206 fmol/mg

FIG. 18

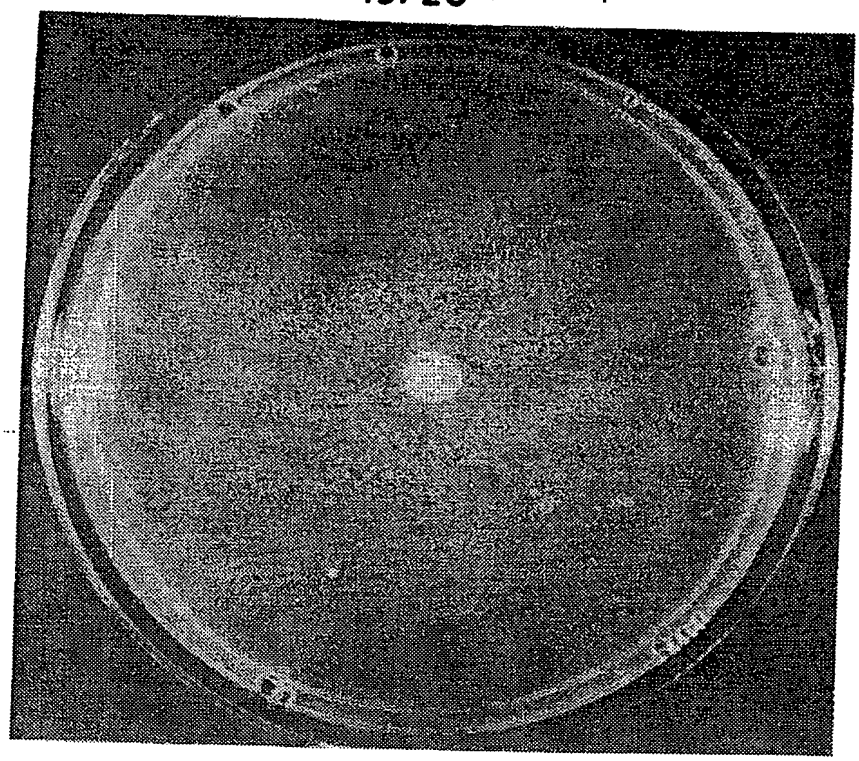


FIG. 19A

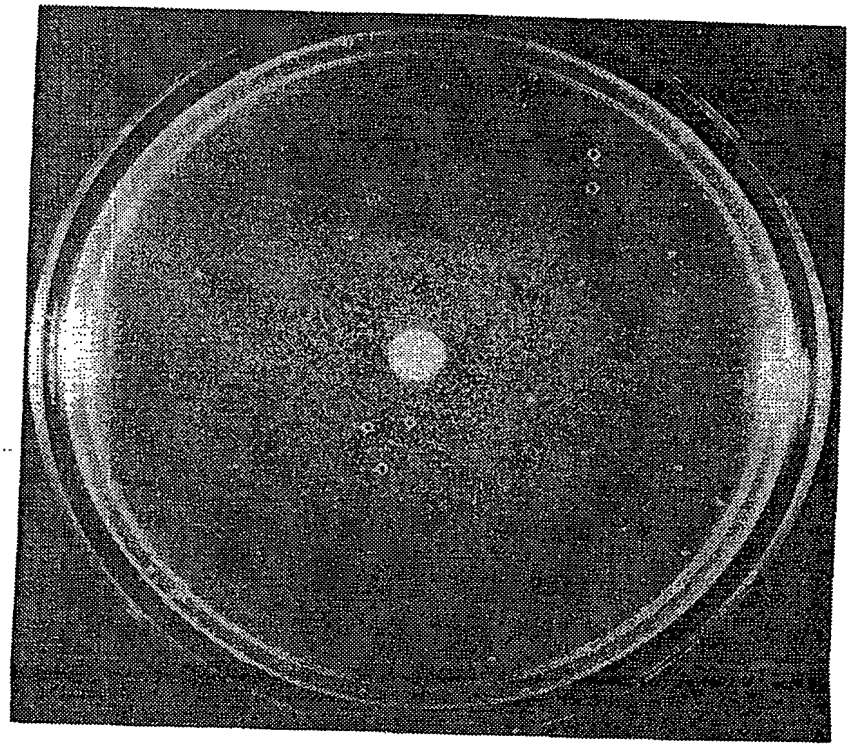


FIG. 19B

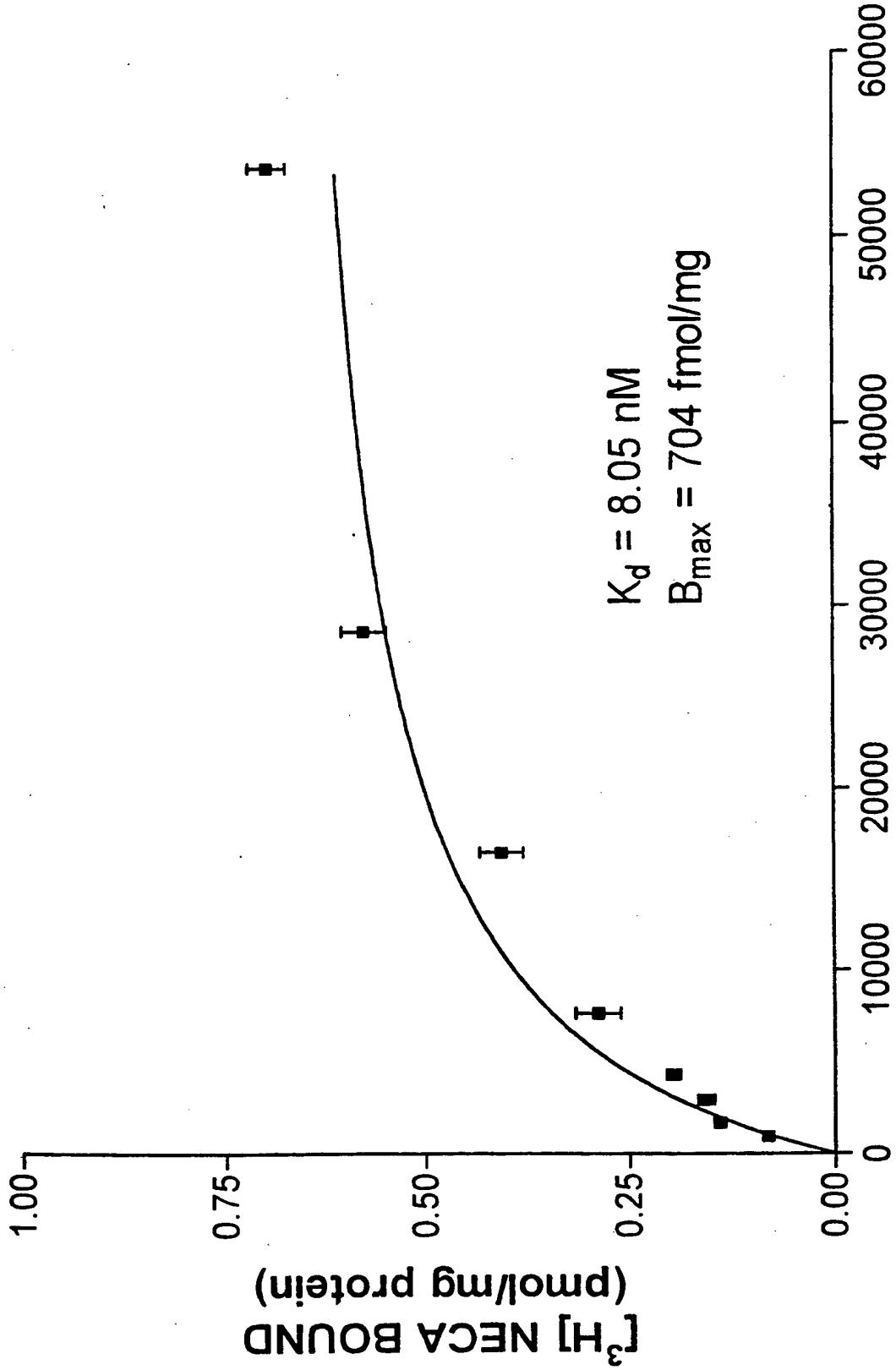

[^{3}H] NECA BOUND
(pmol/mg protein)
1.00
0.75
0.50
0.25
0.00
0
10000
20000
30000
40000
50000
60000
[^{3}H] NECA, pM
K_d = 8.05 nM
B_{max} = 704 fmol/mg

FIG. 20

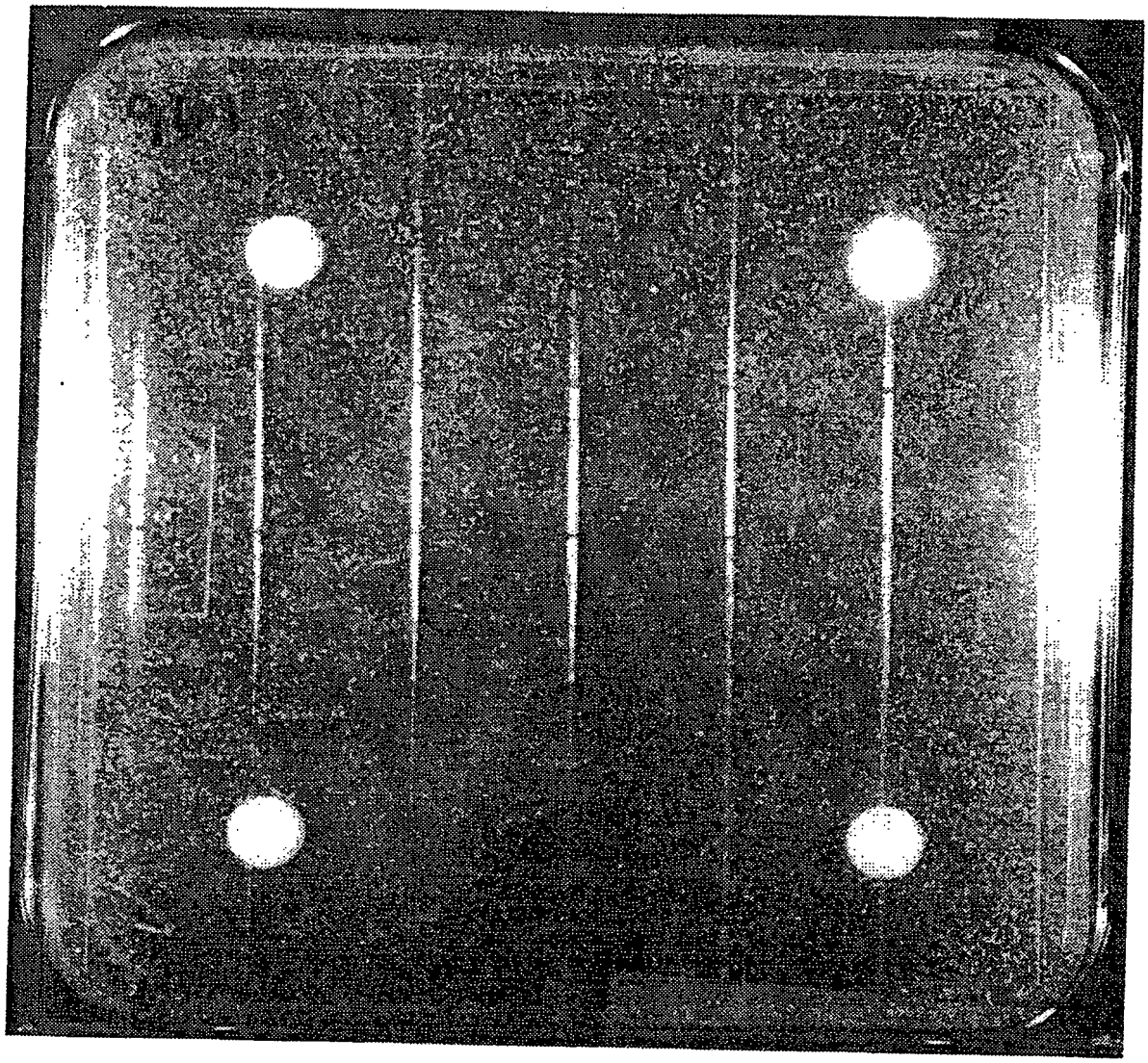


FIG. 21

A

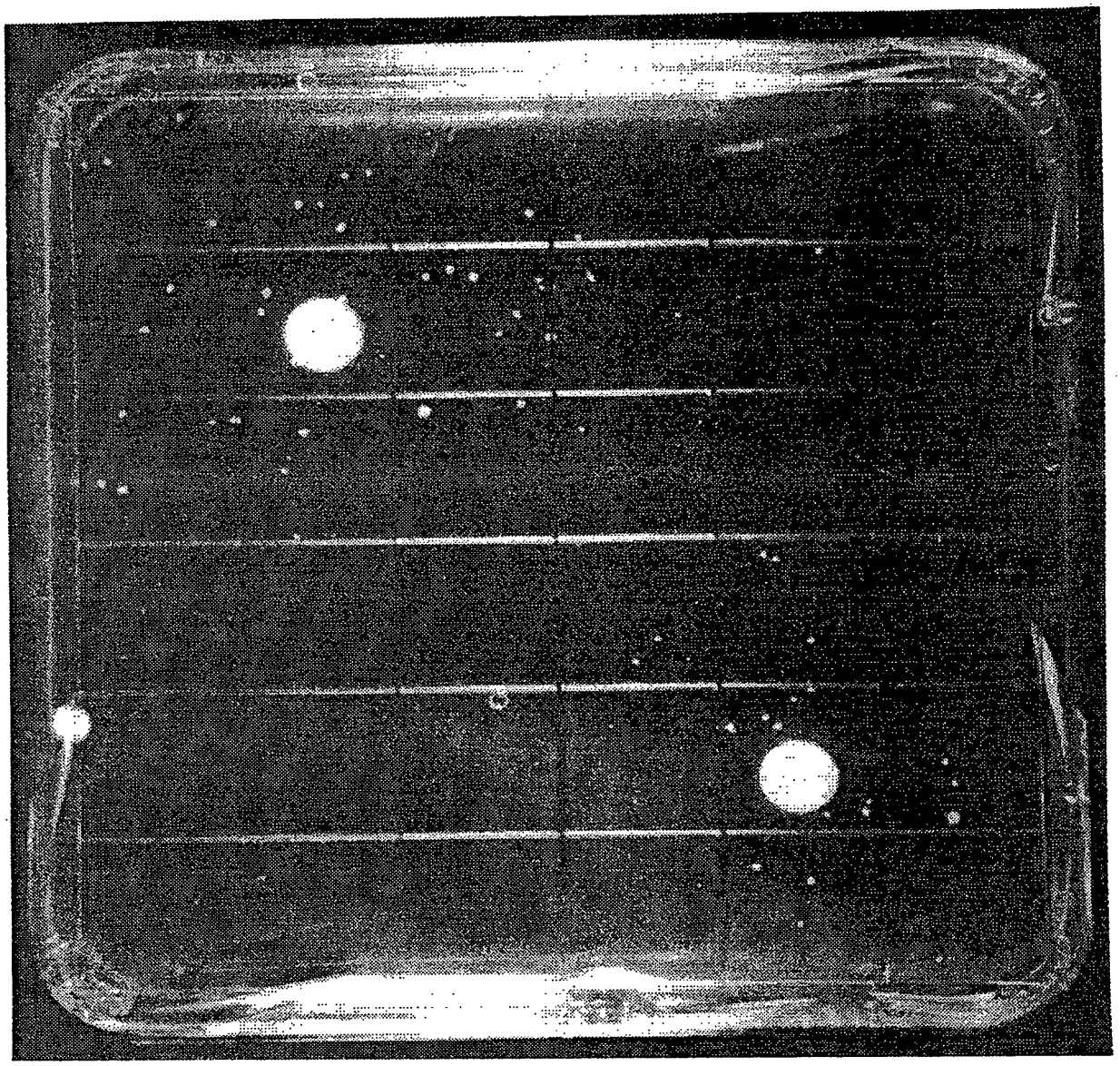


FIG. 22 B

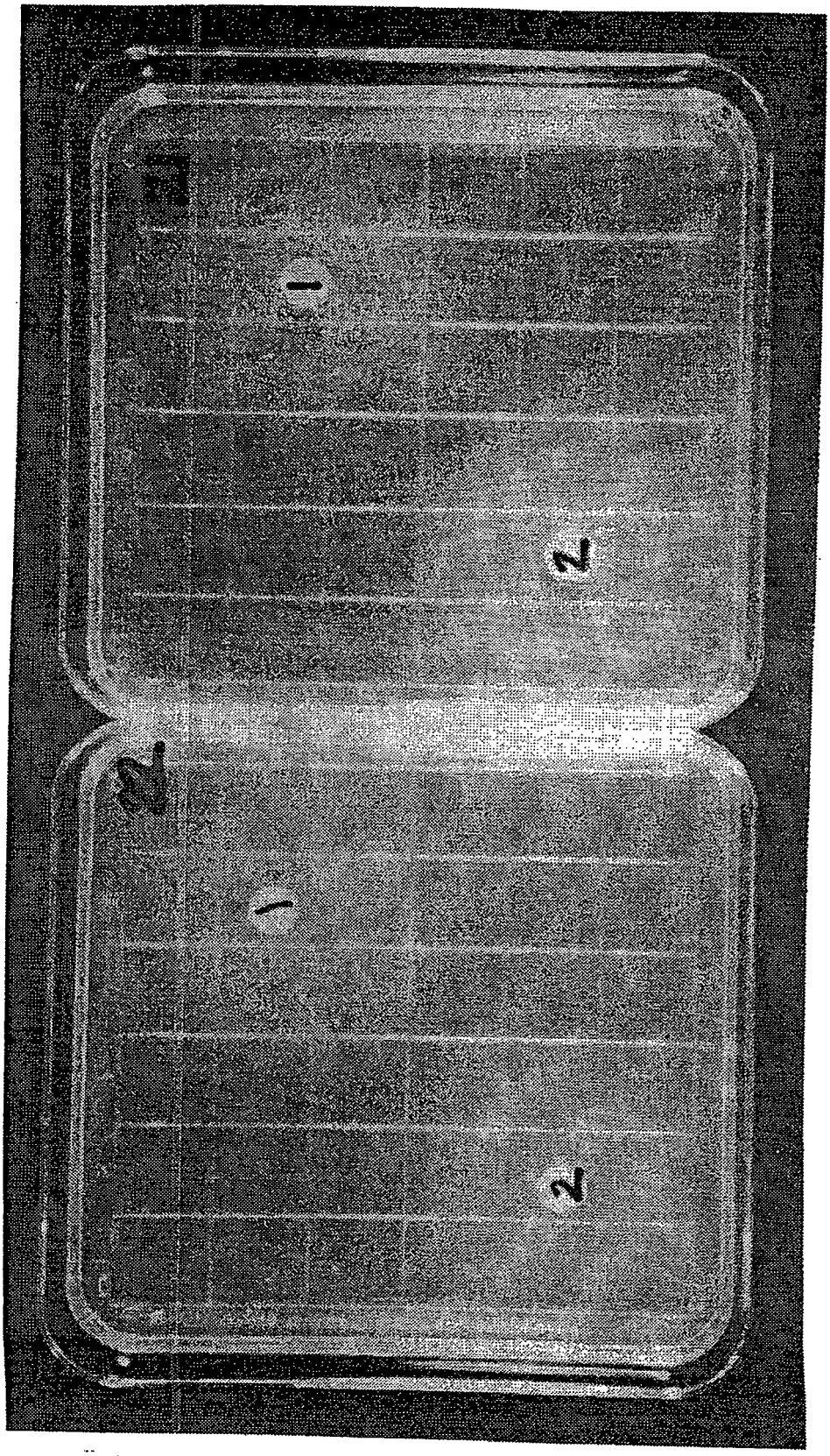


FIG. 23

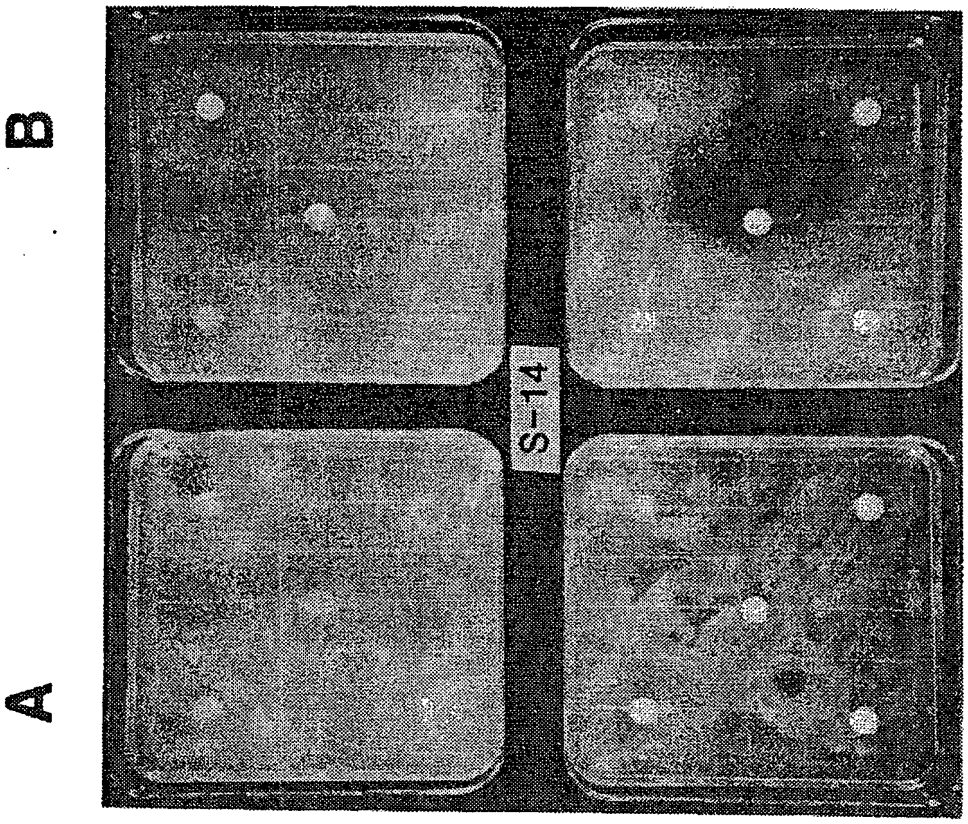


FIG. 24

FIG. 25B

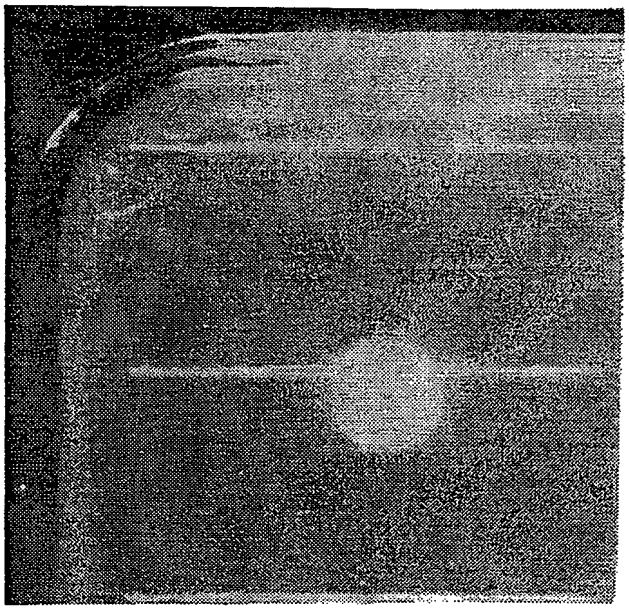


FIG. 25A

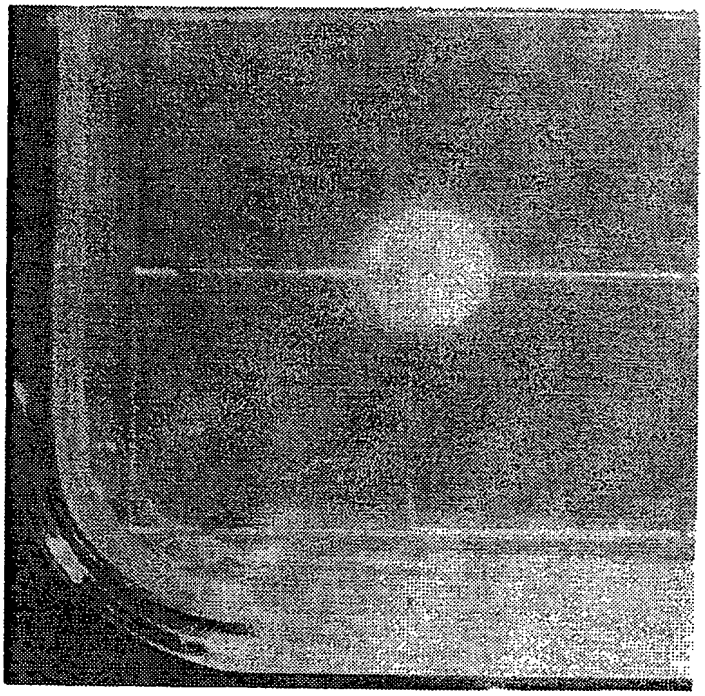


FIG. 26B

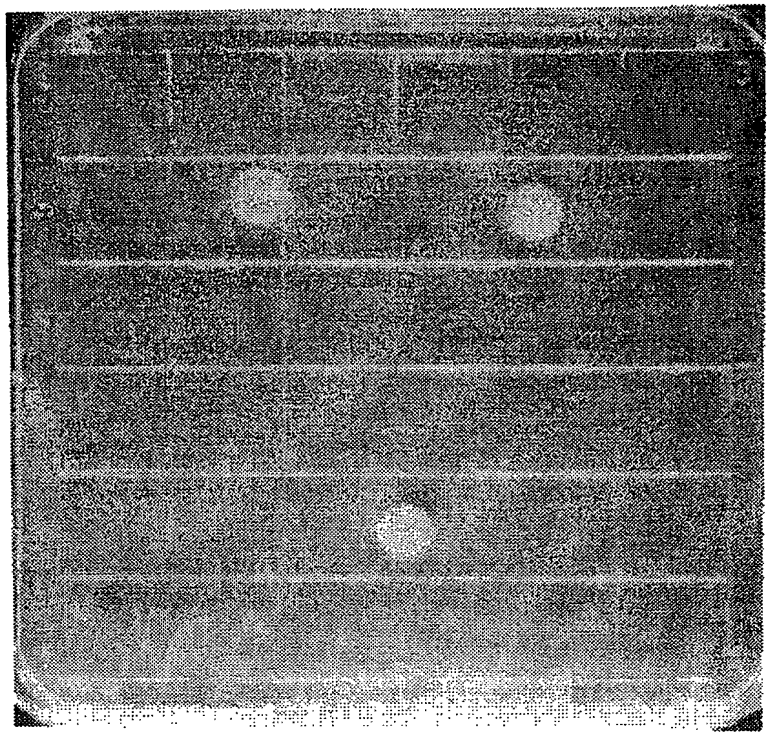


FIG. 26A

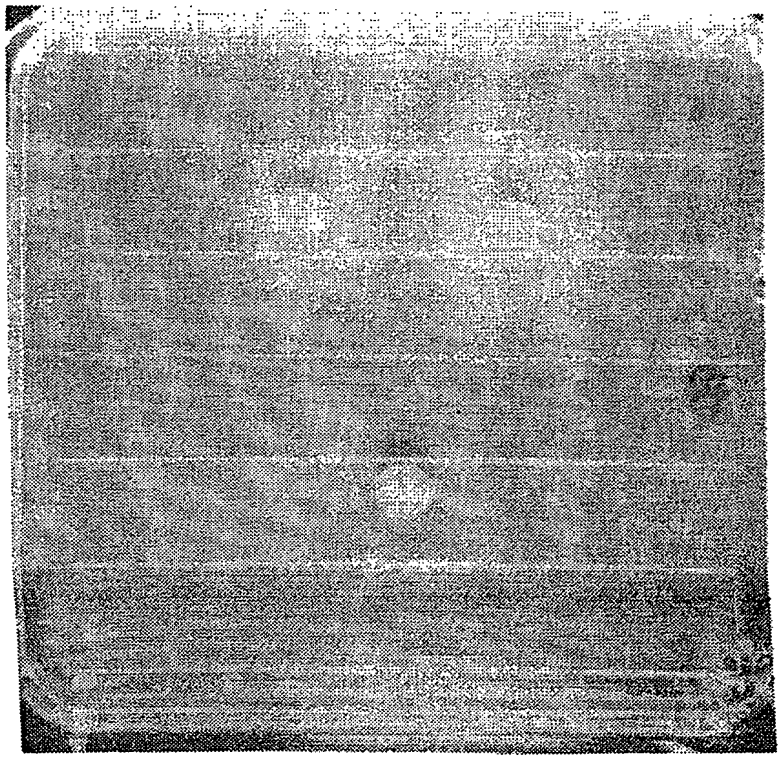
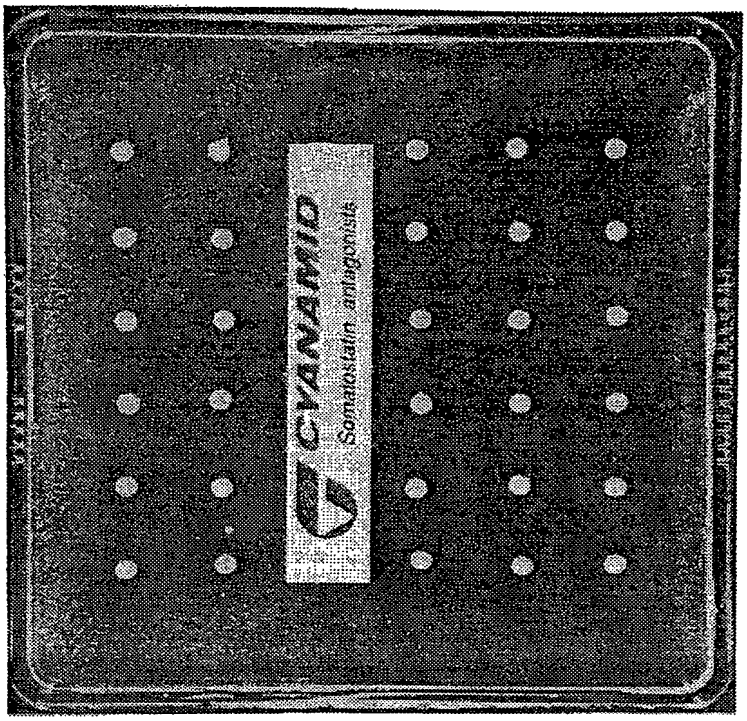
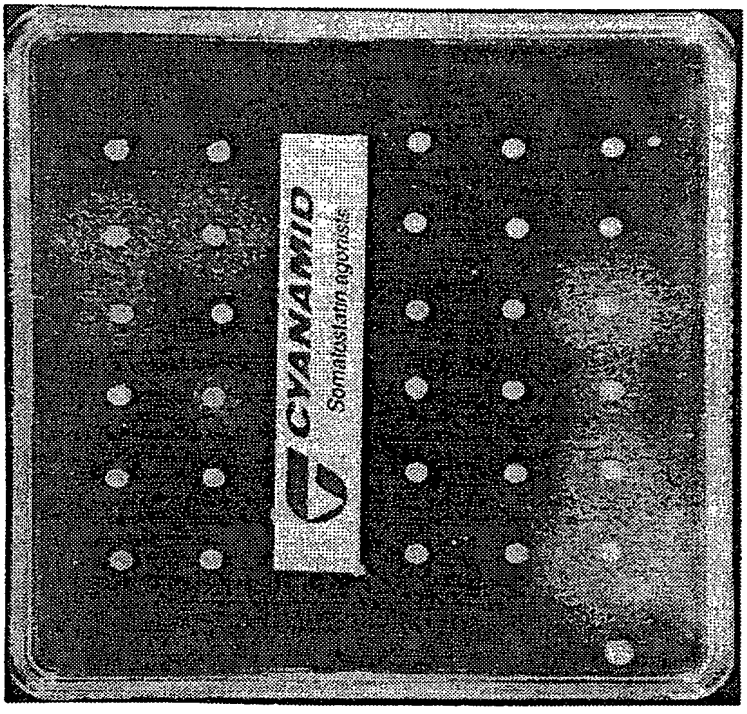


FIG. 27B



CL compounds (10 µl of 10 mg/ml solution) applied to each position.

FIG. 27A



CL compounds (10 µl of 10 mg/ml solution) applied to each position.

FIG. 28B

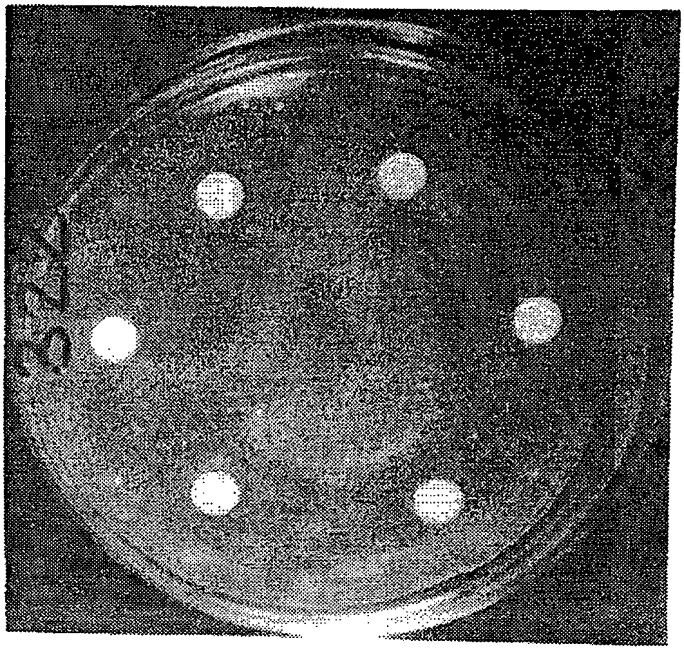
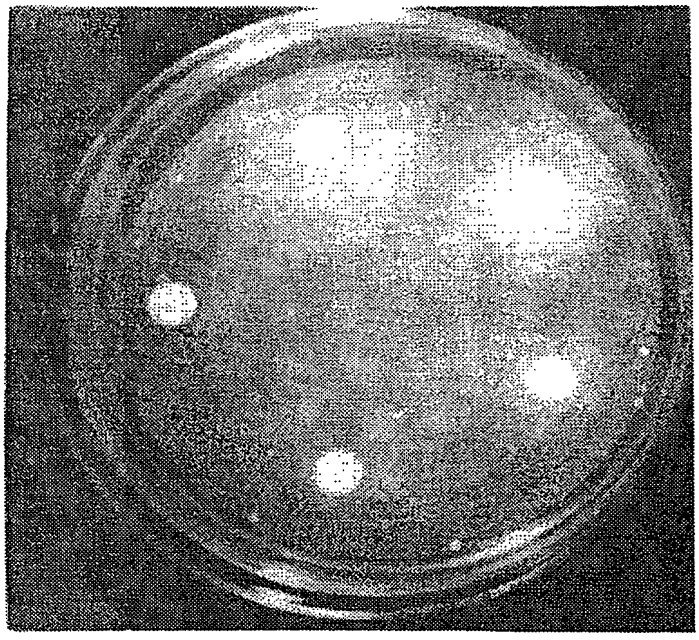


FIG. 28A



INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 95/02075

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N15/81 C12N1/19 C07K14/72 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 05244 (DUKE UNIVERSITY) 2 April 1992 see page 2, line 16 - page 3, line 14 see page 4, line 35 - page 5, line 8 see page 5, line 29 - page 11, line 8; examples ---	1-6,15, 16, 20-22, 24-28
X	WO,A,91 12273 (ZYMOGENETICS, INC.) 22 August 1991 see page 3, line 32 - page 7, line 24 -----	24,25

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 June 1995	04. 07. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

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INTERNATIONAL SEARCH REPORT

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9205244	02-04-92	AU-B-	652576	01-09-94
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		CA-A-	2092717	14-03-92
		EP-A-	0548165	30-06-93
		JP-T-	6500693	27-01-94
WO-A-9112273	22-08-91	US-A-	5284746	08-02-94
		AU-A-	7333691	03-09-91
		EP-A-	0594594	04-05-94
		JP-T-	5504258	08-07-93

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60/061,268	7 October 1997 (07.10.97)	US

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(54) Title: CONSTITUTIVELY ACTIVATED SEROTONIN RECEPTORS

(57) Abstract

Mutations have been discovered in mammalian G protein–coupled serotonin 5–HT_{2A} and 5–HT_{2C} receptors which render the mutated receptors constitutively active. An alignment methodology based on the highly conserved sixth transmembrane domain has been discovered for the monoamine receptors which accurately predicts the amino acid position in the third intracellular loop which, when mutated, produces constitutive activation of the receptor. Constitutive activation of the G protein–coupled serotonin receptors has been shown by the demonstration of an enhanced affinity and potency for serotonin, by increased basal activity of the second messenger system in the absence of agonist, and by reduction of the basal second messenger activity by inverse agonists.

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BNSDOCID: <WO 9838217A1 I_>

CONSTITUTIVELY ACTIVATED SEROTONIN RECEPTORS

The benefit of U.S. Provisional Application No. 60/039,465 filed February 27, 1997, and U.S. Provisional Application No. 60/061,268 filed October 7, 1997 is claimed for this application.

BACKGROUND OF THE INVENTION

Field Of The Invention

The present invention relates generally to the field of transmembrane receptors, more particularly to seven segment transmembrane G protein-coupled receptors, and most particularly to the serotonin (5-HT) receptors. Through genetic mutational techniques, the amino acid sequences of the native 5-HT_{2A} and 5-HT_{2C} receptors have been modified so that the receptors exist in a constitutively activated state exhibiting both a greater response to agonists and a coupling to the G Protein second messenger system even in the absence of agonist. A method for constitutively activating G protein-coupled 5-HT receptors in general is also disclosed.

Description Of Related Art

The research interest in G protein-coupled cell surface receptors has exploded in recent years as it has been apparent that variants of these receptors play a significant role in the etiology of many severe human diseases. These receptors serve a diverse array of signalling pathways in a wide variety of cells and tissue types. Indeed, over the past 20 years, G protein-coupled receptors have proven to be excellent therapeutic targets with the development of several hundred drugs directed towards activating or deactivating them.

G protein-coupled receptors form a superfamily of receptors which are related both in their structure and their function. Structurally the receptors are large macromolecular proteins embedded in and spanning the cell membrane of the receiving cell and are distinguished by a common structural motif. All the receptors have seven domains of between 22 to 24 hydrophobic amino acids forming seven α helixes arranged in a bundle which span the cell membrane substantially perpendicular to the cell membrane. The transmembrane helixes are joined by chains of hydrophilic amino acids. The amino terminal and three connecting chains extend into the extracellular environment while the carboxy

terminal and three connecting chains extend into the intracellular environment. Signalling molecules are believed to be recognized by the parts of the receptor which span the membrane or lie on or above the extracellular surface of the cell membrane. The third intracellular loop joining helixes five and six is thought to be the most crucial domain involved in receptor/G protein coupling and responsible for the receptor selectivity for specific types of G proteins.

Functionally, all the receptors transmit the signal of an externally bound signalling molecule across the cell membrane to activate a heterotrimeric transducing protein which binds GDP (guanosine diphosphate). Upon activation, the bound GDP is converted to GTP (guanosine triphosphate). The activated G protein complex then triggers further intracellular biochemical activity. Different G proteins mediate different intracellular activities through various second messenger systems including, for example, 3'5'-cyclic AMP (cAMP), 3'5'-cyclic GMP (cGMP), 1,2-diacylglycerol, inositol 1,4,5-triphosphate, and Ca^{2+}. Within the human genome, several hundred G protein-coupled receptors have been identified and endogenous ligands are known for approximately 100 of the group. While the seven transmembrane motif is common among the known receptors, the amino acid sequences vary considerably, with the most conserved regions consisting of the transmembrane helixes.

Binding of a signalling molecule to a G protein-coupled receptor is believed to alter the conformation of the receptor, and it is this conformational change which is thought responsible for the activation of the G protein. Accordingly, G protein-coupled receptors are thought to exist in the cell membrane in equilibrium between two states or conformations: an "inactive" state and an "active" state. In the "inactive" state (conformation) the receptor is unable to link to the intracellular transduction pathway and no biological response is produced. In the altered conformation, or "active" state, the receptor is able to link to the intracellular pathway to produce a biological response. Signalling molecules specific to the receptor are believed to produce a biological response by stabilizing the receptor in the active state.

Discoveries over the past several years have shown that G protein-coupled receptors can also be stabilized in the active conformation by means other than

binding with the appropriate signal molecule. Four principal methods have been identified: 1) molecular alterations in the amino acid sequence at specific sites; 2) stimulation with anti-peptide antibodies; 3) over-expression in in vitro systems; and 4) over-expression of the coupling G proteins. These other means simulate the stabilizing effect of the signalling molecule to keep the receptor in the active, coupled, state. Such stabilization in the active state is termed "constitutive receptor activation".

Several features distinguish the constitutively activated receptors. First, they have an affinity for the native signalling molecule and related agonists which is typically greater than that of the native receptors. Second, where several known agonists of varying activity (to the native receptor) were known, it was found that the greater the initial activity of the agonist, the greater was the increase in its affinity for the constitutively activated receptor. Third, the affinity of the constitutively activated receptor for antagonists is not increased over the affinity for the antagonist of the native receptor. Fourth, the constitutively activated receptors remain coupled to the second messenger pathway and produce a biological response even in the absence of the signalling molecule or other agonist.

The importance of constitutively activated receptors to biological research and drug discovery cannot be overstated. First, these receptors provide an opportunity to study the structure of the active state and provide insights into how the receptor is controlled and the steps in receptor activation. Second, the constitutively active receptors allow study of the mechanisms by which coupling to G proteins is achieved as well as how G protein specificity is determined. Third, mutated constitutively active receptors are now recognized in disease states. Study of constitutively activated receptors has demonstrated that many mutations may lead to constitutive activation and that a whole range of activation is possible.

Fourth, the existence of constitutively active receptors provides a novel screening mechanism with which compounds which act to increase or decrease receptor activity can be identified and evaluated. Such compounds may become lead compounds for drug research. Finally, studying the affect of classical antagonists

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(compounds previously identified as, in the absence of agonist, binding to the receptor but causing no change in receptor activitiy, and, in the presence of agonist, competitively decreasing the activity of a receptor) and other drugs used as treatments on the constitutively active receptors has led to the discovery that there are compounds, inverse agonists, which decrease the constitutive activity of the active state of the receptors but which have no or little affect on the inactive state. The difference between antagonists, which act on the inactive state, and inverse agonists, which act on the active state, is only discernable when the receptor exhibits constitutive activity. These inverse agonists, identifiable with constitutively active receptors, present an entirely new class of potential compounds for drug discovery.

About 10 years ago, it was recognized that neurotransmitter receptors can be divided into two general classes depending on the rapidity of their response. Fast receptors were identified with ion channels and mediate millisecond responses while slower receptors were identified with G protein-coupled receptors. These G protein-coupled receptors include certain subtypes of the adrenergic as well as the muscarinic cholinergic (M1 - M5), dopaminergic (D1 - D5), serotonergic (5-HT1, 5-HT2, 5-HT4 - 5-HT7) and opiate (δ, κ, and μ) receptors. Each of these G protein-coupled neurotransmitter receptors has been associated with profound changes in mental activity and functioning, and it is believed that abnormal activity of these receptors may contribute to certain psychiatric disorders. Consequently, the elucidation of the mechanism of action of these receptors has been the focus of vigorous research efforts.

Serotonin receptors are of particular importance. Serotonin-containing cell bodies are found at highest density in the raphe regions of the pons and upper brain stem. However, these cells project into almost all brain regions and the spinal column. Serotonin does not cross the blood-brain barrier and is synthesized directly in neurons from L-tryptophan. In the CNS serotonin is thought to be involved in learning and memory, sleep, thermoregulation, motor activity, pain, sexual and aggressive behaviors, appetite, neuroendocrine regulation, and biological rhythms. Serotonin has also been linked to pathophysiological conditions such as anxiety, depression, obsessive-compulsive disorders,

schizophrenia, suicide, autism, migraine, emesis, alcoholism and neurodegenerative disorders. Presently several drugs are used to modify serotonin receptors: 1) 5-HT1: sumatriptan for treatment of migraine, ipsapirone and buspirone for treatment of anxiety; 2) 5-HT2: clozapine and risperidone for treatment of schizophrenia; and 3) 5-HT3: odanestron for the prevention of emesis in chemotherapy.

To date, fourteen serotonin receptors have been identified in 7 subfamilies based on structural homology, second messenger system activation, and drug affinity for certain ligands. The 5-HT_2 subfamily is divided into 3 classes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. 5-HT_{2A} and 5-HT_{2C} receptor antagonists are thought to be useful in treating depression, anxiety, psychosis, and eating disorders. 5-HT_{2A} and 5-HT_{2C} receptors exhibit 51% amino acid homology overall and approximately 80% homology in the transmembrane domains. The 5-HT_{2C} receptor was cloned in 1987 and led to the cloning of the 5-HT_{2A} receptor in 1990. Studies of the 5-HT_{2A} receptor in recombinant mammalian cell lines revealed that the receptor possessed two affinity states, high and low. Both the 5-HT_{2A} and 5-HT_{2C} receptors are coupled to phospholipase C and mediate responses through the phosphatidylinositol pathway. Studies with agonists and antagonists display a wide range of receptor responses suggesting that there is a wide diversity of regulatory mechanisms governing receptor activity. The 5-HT_{2A} and 5-HT_{2C} receptors have also been implicated as the site of action of hallucinogenic drugs.

Much of the knowledge about the structure of G protein-coupled receptors has come from the study of the β_2-adrenergic receptor. Over the last several years, site-directed mutagenesis has been used to try to determine the amino acid residues important for ligand binding in both the β_2-adrenergic and 5-HT_{2A} receptors. In addition, studies have suggested that in a native (inactive) state of G protein-coupled receptors, the third intracellular loop is tucked into the receptor and is not available for interaction with the G protein. A change of receptor conformation (active) results in the availability or exposure of the C-terminal region of the third intracellular loop.

In 1990 Cotecchia et al.[1] were studying the G protein specificity determining characteristics of the third intracellular loop by creating chimeric

receptors in which the third intracellular loops had been exchanged between the α_1-adrenergic receptor and the β_2-adrenergic receptor. The specific G protein coupled activation was essentially switched between the two receptors. While attempting to determine which portions of the loop were responsible for the specificity, Cotecchia et al. discovered an unexpected phenomena; namely that the modification in the third intracellular loop of the α_1-adrenergic receptor of three residues, Arg288, Lys290, and Ala293, created a mutant receptor with two orders of magnitude greater affinity for agonist and which coupled to the second messenger system even in the absence of agonist. These modifications were made in the carboxy end of the third cytoplasmic loop adjacent to the sixth transmembrane helix. The changes responsible for this increase were isolated to either a Ala293 → Leu or a Lys290 → His mutation. Thus, a constitutively active state of a G protein-coupled neuroreceptor had been created. Subsequently, Kjelsberg et al.[2] demonstrated that mutation of the amino acid at position 293 in the α_{1B}-adrenergic receptor to any other of the 19 amino acids also produced a constitutively active state. Subsequently, mutations in the β_2-adrenergic receptor near the carboxy end of the third cytoplasmic loop have also been shown by Samama et al.[3] to constitutively activate this receptor.

When foci resulting from constitutively active α_{1B}-adrenergic receptors were injected into nude mice, tumor formation occurred. Over the past 5 years, since the discovery that several thyroid adenomas contained mutations of the thyroid stimulating hormone (TSH) receptor, constitutively activated receptors have been found associated with several human disease states. The mutations responsible for these disease states have been found in the transmembrane domains and intracellular loops. For the TSH receptor, mutations at 13 different amino acid positions have been found in the transmembrane domain, the third intracellular loop, and the second and third extracellular loops. Clearly, constitutively activating mutations are not limited to the third intracellular loop and the critical site for constitutive activation varies with each G protein-coupled receptor. The importance of the initial observations was well stated in Cotecchia et al.[1]: "Such mutations might not only help to illuminate the biochemical mechanisms involved in receptor-G protein coupling but also provide models for how point mutations

might activate potentially oncogenic receptors."

In light of the above referenced discoveries, the importance and utility of discovering other constitutively activated neuronal receptors cannot be understated. However, the hope that other neuronal receptors could be easily and readily mutated to a constitutively active form by mutations in the third cytoplasmic loop was destroyed by the report of Burstein et al.[4] in 1995 of a comprehensive mutational approach to the G protein coupled M5 muscarinic acetylcholine receptor. In that approach, Burstein et al. had randomly and comprehensively mutated the C-terminal region of the third intracellular loop of the M5 muscarinic acetylcholine receptor, but no constitutive activating mutations were found.

Definition: CONSTITUTIVELY ACTIVATED RECEPTOR shall mean a G protein-coupled receptor which: 1) exhibits an increase in basal activity of the second messenger pathway in the absence of agonist above the level of activity observed in the wild type receptor in the absence of agonist; 2) may exhibit an increased affinity and potency for agonists; 3) exhibits an unmodified or decreased affinity for antagonists; and 4) exhibits a decrease in basal activity by inverse agonists.

SUMMARY OF THE INVENTION

Constitutively active forms of the rat 5-HT_{2A} and 5-HT_{2C} serotonin receptors have been obtained by a site-directed mutational method that will permit the constitutive activation of all mammalian G protein-coupled serotonin receptors. An amino acid position that will lead to a successful mutation in the serotonin receptor may be identified by alignment of the serotonin receptor against the amino acid sequence of the α_{1B}-adrenergic receptor. Mutating the amino acid in the serotonin receptor which corresponds to the most sensitive position in the α_{1B}-adrenergic receptor, alanine 293, yields a constitutively active serotonin receptor. A strongly constitutively active serotonin receptor is achieved when the mutation in the serotonin receptor is to one of the amino acids which produces the highest level of basal activation in constitutively activated α_{1B}-adrenergic receptors. Successful constitutive activation of the serotonin receptor can be shown by increased high basal levels of second messenger activity in the absence of agonist, increased affinity and potency for agonists, and an unmodified or

decreased affinity for antagonists. While standard methods of site-directed mutagenesis may be employed, the careful placement of restriction sites in the primer permits the more rapid and direct determination of the clone containing the desired mutated receptor.

It is the object of this invention to provide a general methodology for obtaining constitutively active forms of the G protein-coupled mammalian monoamine receptors.

It is a further object of this invention to provide a general methodology for obtaining constitutively active forms of the G protein-coupled mammalian serotonin receptors.

It is another object of this invention to provide a constitutively active 5-HT_{2A} serotonin receptor.

It is a further object of this invention to provide a constitutively active 5-HT_{2C} serotonin receptor.

Yet another object of this invention is to provide a method for rapidly identifying the clone containing the desired mutated receptor.

These and other achievements of the present invention will become apparent from the detailed description which follows.

DESCRIPTION OF THE FIGURES

Figure 1A shows the full DNA sequence for the rat 5-HT_{2A} serotonin receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. Figure 1B shows the translated amino acid sequence for the rat 5-HT_{2A} receptor.

Figure 2A shows the full DNA sequence for the rat 5-HT_{2C} serotonin receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. Figure 2B shows the translated amino acid sequence for the rat 5-HT_{2C} receptor.

Figure 3A shows the full DNA sequence for the rat α_{1B}-adrenergic receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. Figure 3B shows the translated amino acid sequence for the rat α_{1B}-adrenergic receptor.

Figure 4 shows the amino acid sequences for part of the C-terminal third

intracellular loop and transmembrane domain VI for the 5-HT_{2A} and 5-HT_{2C} receptors aligned opposite the corresponding part of the α_{1b}-adrenergic receptor with numerals representing the amino acid positions in each receptor.

Figure 5 shows a schematic outline of the 5-HT_{2A} site-directed mutagenesis procedure.

Figure 6 shows a schematic outline of the 5-HT_{2C} site-directed mutagenesis procedure.

Figure 7 shows the competition curves of 5-HT for ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors. 0.5nM ^{3}H-ketanserin was used to label the native and mutant receptors transiently transfected in COS-7 cells.

Figure 8 shows the radioligand binding data of ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors in the presence of agonists and antagonists. 0.5 nM ^{3}H-ketanserin was used to label the native and mutant 5-HT_{2A} receptors expressed in COS-7 cells.

Figure 9 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT_{2A} receptors. IP production assays were performed using anion-exchange chromatography. The data are expressed as percent of maximal IP stimulation produced by 10 μM 5-HT.

Figure 10 shows the basal activity and 5-HT stimulation of the native and mutant 5-HT_{2A} receptors. IP levels were measured in COS-7 cells with vector alone, native 5-HT_{2A} receptors, or mutant 5-HT_{2A} receptors. The data are expressed as dpms of IP stimulation minus basal levels of IP produced by vector. Basal activity of vector alone was typically 400 dpms.

Figure 11 shows a saturation analysis of ^{3}H-ketanserin labeled native and cys → lys mutant receptors. Bmax values were determined by a BCA assay.

Figure 12 shows the competition curves of 5-HT for ^{3}H-mesulergine labeled native and mutant 5-HT_{2C} receptors. 1 nM ^{3}H-mesulergine was used to label the native and mutant receptors transiently transfected in COS-7 cells.

Figure 13 shows the radioligand binding analysis of native and mutant 5-HT_{2C} receptors. Native and mutant 5-HT_{2C} receptors expressed in COS-7 cells were labeled with 1 nM ^{3}H-mesulergine. 5-MT = 5-methoxytryptamine.

Figure 14 shows the 5-HT stimulation of IP production in COS-7 cells

transfected with the ser → lys or ser → phe mutated receptors. Cells were labeled with ^{3}H-myoinositol and challenged with 5-HT (0.1 nM - 10 nM). Total IP production was measured by anion exchange chromatography.

Figure 15 shows the EC_{50} values for the 5-HT stimulation of IP production in COS-7 cells transfected with native, mutant ser → lys receptor, and mutant ser → phe receptor. Figure 15 also shows the results of ^{3}H-mesulergine saturation analyses. Saturation experiments were performed using ^{3}H-mesulergine (0.1 nM - 5.0 nM).

Figure 16 shows the effect of the ser → lys and ser → phe mutations on basal levels of IP production by the mutated 5-HT_{2C} receptors. IP levels were measured in COS-7 cells with vector alone, native 5-HT_{2C} receptors, or mutant 5-HT_{2C} receptors. The data are expressed as dpms of IP stimulation minus basal levels of IP produced by vector.

Figure 17 shows the inverse agonist activity of spiperone and ketanserin on the mutated constitutively active 5-HT_{2A} cys → lys receptor. Parallel transfections with the native 5-HT_{2A} receptor were performed to determine native basal activity which was then subtracted from the mutant receptor basal activity to determine constitutive stimulation.

Figure 18 shows the inverse agonist activity of chlorpromazine, haloperidol, loxapine, spiperone, clozapine and risperidone on the mutated constitutively active 5-HT_{2A} cys → lys receptor.

Figure 19 shows the inverse agonist activity of mianserin and mesulergine on the mutated constitutively active 5-HT_{2C} ser → lys receptor both in the presence and absence of 5-HT.

Figure 20A sets forth the full DNA sequence for the human 5-HT_{2A} serotonin receptor with the translated codons underlined. The sixth transmembrane domain conserved sequence of WxPFFI is indicated with block letters. Figure 20B shows the translated amino acid sequence for the human 5-HT_{2A} receptor.

Figure 21A sets forth the full DNA sequence for the human 5-HT_{2C} serotonin receptor with the translated codons underlined. The sixth transmembrane domain conserved sequence of WxPFFI is indicated with block

letters. Figure 21B shows the translated amino acid sequence for the human 5-HT_{2C} receptor.

Figure 22 is the amino acid sequence of the 5-HT_{2A} cys → lys mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 23 is the DNA sequence of the 5-HT_{2A} cys → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 24 is the DNA sequence of the 5-HT_{2A} cys → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the two bases which were mutated to create the Sca1 site are shown as larger outlined letters and are indicated with arrows.

Figure 25 is the amino acid sequence of the 5-HT_{2A} cys → arg mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 26 is the DNA sequence of the 5-HT_{2A} cys → arg mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 arginine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 27 is identical to Figure 26 since the AGG mutation introduced for arginine creates an MnlI restriction site by itself at #319.

Figure 28 is the amino acid sequence of the 5-HT_{2A} cys → glu mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 29 is the DNA sequence of the 5-HT_{2A} cys → glu mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 30 is the DNA sequence of the 5-HT_{2A} cys → glu mutant receptor

including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the additional base which was mutated to create the Rsa1 site is shown as a larger outlined letter and is indicated with an arrow.

Figure 31 is the amino acid sequence of the 5-HT_{2c} ser → lys mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 32 is the DNA sequence of the 5-HT_{2c} ser → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 33 is the DNA sequence of the 5-HT_{2c} ser → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the base which was mutated to create the Sca1 site is shown as a larger outlined letter and is indicated with an arrow.

Figure 34 is the amino acid sequence of the 5-HT_{2c} ser → phe mutant receptor with the mutated amino acid shown as a larger outlined letter.

Figure 35 is the DNA sequence of the 5-HT_{2c} ser → phe mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated.

Figure 36 is the DNA sequence of the 5-HT_{2c} ser → phe mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition the base which was mutated to create the Sca1 site is shown as a larger outlined letter and is indicated with an arrow.

DETAILED DESCRIPTION OF THE INVENTION

Despite the disappointing results obtained by Burstein in mutating positions in the third intracellular loop of the M5 muscarinic acetylcholine receptor, the present inventive efforts focused on finding mutations at the carboxy end of the third intracellular loop near the sixth transmembrane helix in the serotonin receptors. DNA and amino acid sequences for rat 5-HT_{2A} and 5-HT_{2C} serotonin receptors were obtained from GeneBank as was the DNA and amino acid sequence for the α_{1B}-adrenergic receptor. Figures 1, 2, and 3 list the full DNA and translated amino acid sequences for these receptors.

Receptor Alignment:

As noted above, Cotecchia et al. had identified amino acid position number 293 in the third intracellular loop adjoining the sixth transmembrane domain in the α_{1B}-adrenergic receptor as a critical position, mutation of which lead to constitutive activity. However, the length of the serotonin receptors is different than the α_{1B}-adrenergic receptor, and even had they been the same, matching the ends would not necessarily provide a structural or functional match. What was important was to find an alignment method which made sense in terms of locating the equivalent functional site to position 293 of the α_{1B}-adrenergic receptor in the serotonin receptors.

A meaningful alignment method has been discovered based upon the fact that the transmembrane domains are highly conserved in G protein-coupled receptors. A series of conserved amino acid positions were identified in the sixth transmembrane domain which permit alignment of the transmembrane domain and the adjacent third intracellular loop between receptors. In Figure 5 the conserved sixth transmembrane domain amino acid sequence WxPFFI (x may be variable) has been used to align the three receptors. Alignment using this sequence also aligns the LGIV sequence found at the intracellular beginning of the sixth transmembrane domain which is connected to the third intracellular loop. This alignment indicates that in the 5-HT_{2A} receptor the cysteine at position #322 corresponds to the alanine at position #293 in the α_{1B}-adrenergic receptor. In the 5-HT_{2C} receptor, the corresponding amino acid is a serine at position #312.

It should be noted that position 293 is not the only position in the α_{1B}-

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adrenergic receptor which, when mutated, produced a constitutively active receptor. While Cotecchia et al.[1] reported that the A293L mutation produced the greatest constitutive activation, they also noted that the K290H mutation also induced dramatic constitutive activity. There are clearly other sites in the third intracellular loop of each of these receptors that can be mutated. In the future, other sites on other receptors may be reported. However, the alignment methodology presented above should serve to permit the structural correlation between different receptors so that information gleaned from one receptor may be utilized to mutate another receptor. However, the evidence presently available suggests that the third position removed from the beginning of the transmembrane domain represented by position 293 in the α_{1B}-adrenergic receptor seems to play a crucial role in the binding and activation of the coupled G protein, and that mutations introduced at that position alter the tertiary structure of the region.

As noted earlier, Kjelsberg et al.[2] further demonstrated that substitution of any of the 19 amino acids at position 293 of the α_{1B}-adrenergic receptor produced constitutive activity. However, the relative activity increased in the following order of amino acids: S, N, D, G, T, H, W, Y, P, V, L, M, Q, I, F, C, R, K, and E. In that study, replacing the native amino acid with amino acids having long basic or acidic side chains produced the greatest degree of constitutive activity, while amino acids with aromatic substituents produced an intermediate degree of constitutive activity. It is proposed that this order, with minor variations, exists for most G protein-coupled receptors due to the importance of the third position removed from the beginning of the transmembrane domain. A reasonable starting place for mutating receptors should therefore involve mutation to one of the amino acids at the most active end of the above list. Further, the tertiary structure of the region may be significantly altered by substituting an amino acid with longer side chains or of different polarity from the native amino acid.

Efficient Screening of Mutant Receptors:

When performing site-directed mutagenesis, it is common (and necessary) laboratory practice to fully sequence the cloned receptor to confirm that the mutation has been incorporated. However, because colonies containing the

mutant receptor cannot be distinguished from those that do not, it is necessary to sequence each colony. A method, outlined schematically by way of example in Figure 5 for the 5-HT_{2A} cys → lys receptor mutation and in Figure 6 for the 5-HT_{2C} ser → lys and ser →phe receptor mutations, has been devised that rapidly and easily eliminates most non-mutated colonies, and from those remaining, identifies the mutant colony so that unnecessary sequencing is avoided. A two-pronged approach is used. The first prong is designed to prevent non-mutated vector from being incorporated during the first transformation by digesting the vector. E coli will only incorporate uncut (circular) plasmid DNA. Recognizing the limitations of the first prong, namely, that all restriction digests are not 100% complete so that some of the colonies at the end of the procedure will contain native DNA instead of mutant DNA, the second prong is designed to easily identify among the remaining colonies, those colonies containing the desired mutation after a second transformation.

To begin, a unique restriction site, not occurring in the native amino acid sequence, is incorporated into the mutant. It is possible to introduce the unique restriction site because of the degeneracy of the genetic code. The unique restriction site is ideally located within or near the amino acid(s) which specify the structural mutation which is being introduced into the mutant. Thus, the restriction site can be located on the same mutagenic primer as the structural mutation.

In addition, during the initial annealing, a second primer is used to remove a restriction site specific to the vector being used. When the second strand is synthesized with polymerase and ligase, only the second strand of the vector (the one not containing the mutations) will contain the original vector restriction site. Subsequently, after transformation, the colonies can be treated with the restriction enzyme specific for the vector site and only those resulting from the wildtype vector will be digested. Digested (cut) DNA will not be taken up by E. coli during the second transformation step. The colonies containing the mutated vector will not be digested and will be taken up by E. coli during the final transformation step.

Each resulting colony can be tested to see whether the restriction enzyme,

which recognizes the unique site introduced by the mutated primer, digests the DNA. Only samples from colonies containing the desired mutation will be digested. These colonies can then be sequenced to confirm the insertion of the mutated amino acid. It is unnecessary to sequence colonies whose DNA is not digested by the restriction enzyme. This procedure yields a much more highly efficient method by saving both time and expense of sequencing every colony which results from the transformation experiment.

Measurement of Receptor-Coupled Second Messenger Activation:

In order to measure the stimulation produced through the 5-HT_{2A} and the 5-HT_{2C} receptors, an assay was utilized which measures the accumulation of inositol phosphates, the product that is formed when phosphatidylinositol 4,5-bisphosphate is hydrolyzed to DAG and IP. This assay was established by Berridge and coworkers (1983) in studies of the blowfly salivary glands, and found to be an accurate measurement of the stimulation of phospholipase C through receptor activation. ^{3}H-myoinositol is incorporated into the cell membrane by conversion to phosphatidylinositol 4,5-bisphosphate and upon receptor activation, is cleaved by phospholipase C to yield two products: diacylglycerol and ^{3}H-inositol 1,4,5 triphosphate (IP_3).

Inositol-free media must be used for this assay because unlabeled inositol, which is normally found in many commercially available media, can result in less than maximal incorporation of radiolabeled inositol into the cell membrane, resulting in a reduction in the amount of ^{3}H-IP that would be detected. The ^{3}H-IP is recovered by anion-exchange chromatography in which IP is separated from anion-exchange resin using washes of increasing concentrations of formate.

IP_3 is rapidly hydrolyzed to IP_2 by an inositol triphosphatase which is then converted to IP by inositol bisphosphatase. Because IP_3 is hydrolyzed so quickly, accumulation of IP would be hard to measure unless the cycle of IP to inositol and phosphate is blocked. Lithium is used in this assay to block the enzyme which converts IP to inositol and phosphate (myo-inositol monophosphatase). This ensures that IP levels can accumulate and be experimentally measured and are not undergoing the normal rapid degradation pathway. These experiments are also performed in serum free media in order to remove serotonin that can be found in

serum which would complicate experimental results.

The total IP levels were measured in order to obtain an accurate measurement of the total amount of stimulation that occurred. The actual experimental conditions and concentrations of reagents used in this assay are set forth in the methods and materials sections under each example below.

Example 1: Constitutive Activation Of The 5-HT_{2A} Receptor:

Three separate mutations of the 5-HT_{2A} receptor were made. The cysteine at position 322 was mutated to lysine, glutamate, and arginine.

Materials and Methods For Site-directed Mutagenesis:

The rat 5-HT2A receptor cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen) using EcoR1 (GIBCO). This construct served as the native template for site-directed mutagenesis performed using Clontech's transformer kit. Mutagenic primers (Midland Certified Reagent Company) were designed as follows: the C322K primer was complementary to amino acid nos. 318-329 of the native 5-HT2A cDNA, while changing amino acid no. 322 from cysteine (TGC) to lysine (AAG). The same primer was designed to incorporate a Sca1 restriction site using amino acid nos. 323 and 324 by changing the third base in amino acid no. 323, lysine, from AAG to AAA and the third base in amino acid no. 324, valine from GTG to GTA. The C322E and C322R were designed complementary to amino acid nos. 319-330 of the native 5-HT2A cDNA, while changing amino acid no. 322 from cysteine (TGC) to glutamate (GAG) and arginine (AGG). In the C322E primer, an Rsa1 site was introduced by changing the third base in amino acid no. 324, valine, from GTG to GTA. The C322R mutation in the primer created an Mnl1 site, by itself, at amino acid no. 319. The selection primer, complementary to bases 4,871-4,914 of the pcDNA3 vector, was designed to remove a unique PVUI site by changing base G to T at location 4891. Phosphorylated primers were annealed to 10 ng of alkaline-denatured plasmid template by heating to 65°C for 5 min and cooling slowly to 37°C. Mutant DNA was synthesized using T4 DNA polymerase and ligase (Clontech) by incubating for 1 hr at 37°C, followed by digestion with PVU1 (GIBCO) and transformation of BMH71-18mutS E. coli (Clontech). Plasmid was purified using the Wizard miniprep kit (Promega), digested with PVU1, and used to transform

DH5(E.Coli (GIBCO). Individual colonies were isolated and plasmid DNA was digested with SCA1, MnI1 or Rsa1 to screen for C322K, C322E and C322R mutations, respectively (GIBCO). DNA sequencing (Sequenase version 2.1 kit,USB, ^{35}Sd-ATP, New England Nuclear) was performed to confirm the incorporation of lysine, glutamate, or arginine at amino acid no. 322. Sequencing reactions were run on a 5% acrylamide/bis (19:1) gel (Bio-Rad) for 2 hr at 50°C, dried for 2 hr at 80°C, and exposed on Kodak Biomax MR film for 24 hr at –80°C.

In Figure 22 is shown the amino acid sequence of the 5-HT_{2A} cys → lys mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 23 shows the resulting DNA sequence of the 5-HT_{2A} cys → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT_{2A} cys → lys mutant receptor, Figure 24 shows the two bases, which were mutated to create the Sca1 site, as larger outlined letters and are indicated with arrows.

In Figure 25 is shown the amino acid sequence of the 5-HT_{2A} cys → arg mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 26 shows the resulting DNA sequence of the 5-HT_{2A} cys → arg mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 arginine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. Figure 27 showing the added restriction site is identical to Figure 26 since the arginine mutation to AGG creates, by itself, an MnI1 restriction site at #319.

In Figure 28 is shown the amino acid sequence of the 5-HT_{2A} cys → glu mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 29 shows the resulitng DNA sequence of the 5-HT_{2A} cys → glu mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #322 glutamic acid mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. Figure 30 shows the additional base mutation introduced in amino

acid 324 to create an Rsa1 site. The base mutation is indicted by a larger outlined letter and an arrow.

Cell culture and transfection:

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% fetal bovine serum (Sigma) in 5% CO_2 at 37°C and subcultured 1:8 twice a week. Twenty-four hours before transfection, cells were seeded at 30% confluence in 100-mm dishes for radioligand binding assays or at 10^5 cells per well in 24-well cluster plates for IP production assays. Cells were transfected with native or mutant 5-HT2A cDNA using Lipofectamine (GIBCO). This was accomplished by combining 20 μl of Lipofectamine with 2.5 μg of plasmid per 100-mm dish or 2 μl of Lipofectamine with 0.25 μg of plasmid per well. Transfections were performed in serum-free DMEM for 4 hr at 37°C.

Radioligand binding:

Thirty-six hours after transfection, membranes were prepared from COS-7 cells by scraping and homogenizing in 50mM Tris-HCl/5mM $MgCl_2$/0.5mM EDTA, pH 7.4 (assay buffer), and centrifugation at 10,000xg for 30 min. Membranes were resuspended in assay buffer, homogenized, and centrifuged again. After resuspension in assay buffer, 1-ml membrane aliquots (approximately 10 μg of protein measured by bicinchoninic acid assay) were added to each tube containing 1ml of assay buffer with 0.5nM [^{3}H] ketanserin and competing drugs. 10μM spiperone was used to define non-specific binding. Saturation experiments were performed by using [^{3}H]ketanserin (0.1-5.0nM). Samples were incubated at 23°C for 30 minutes, filtered on a Brandel cell harvester, and counted in Ecoscint cocktail (National Diagnostics) in a Beckman liquid scintillation counter at 40% efficiency.

Phosphatidylinositol hydrolysis:

Inositol phosphate (IP) production was measured using a modified combination of the methods of Berridge et al. (1982) and Conn and Sanders-Bush (1985). In brief, 24 h after transfection, cells were washed with phosphate-buffered saline (PBS) and labeled with 0.25 μCi/well of myo-[^{3}H]inositol (New England Nuclear) in inositol free/serum-free DMEM (GIBCO) for 12 h at 37°C. HPLC analysis of this culture medium, after incubation, has been reported to

contain $<10^{-10}$M 5-HT (Barker et al. 1994). After labeling, cells were washed with PBS and preincubated in inositol-free/serum-free DMEM with 10mM LiCl and 10μM pargyline (assay medium) for 10 min at 37°C. When antagonists were used, they were added during the 10-min preincubation period. 5-HT (Sigma), or assay medium alone, was added to each well and incubation continued for an additional 35 min (Westphal et al., 1995). Assay medium was removed and cells were lysed in 250 μl of stop solution (1 M KOH/18mM sodium borate/3.8mM EDTA) and neutralized by adding 250 μl of 7.5 % HCl. The contents of each well were extracted with 3 volumes of chloroform/methanol (1:2), centrifuged 5 min at 10,000xg, and the upper layer loaded onto a 1-ml AG1-X8 resin (100-200 mesh, Bio-Rad) column. Columns were washed with 10ml of 5 mM myo-inositol and 10ml of 5 mM sodium borate/60mM sodium formate. Total IPs were eluted with 3ml of 0.1 M formic acid/1 M ammonium formate. Radioactivity was measured by liquid scintillation counting in Ecoscint cocktail.

Demonstration of Constitutive Activation:

Constitutive activity of the mutated 5-HT_{2A} receptors is demonstrated by the fact that the mutated receptors exhibit all the hallmark characteristics established for constitutive activation: a showing of increased agonist affinity, increased agonist potency, and coupling to the G protein second messenger system in the absence of agonist.

Figure 7 shows the competition curves of 5-HT for ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors. 0.5nM ^{3}H-ketanserin was used to label the native and mutant receptors transiently transfected in COS-7 cells. While the native receptor demonstrated a relatively low affinity for 5-HT (K_i = 293 nM), the three mutant receptors displayed a high affinity for 5-HT with the cys → lys mutant exhibiting a 12-fold increase in affinity for 5-HT (K_i = 25 nM), the cys → arg mutant exhibiting a 27-fold increase in affinity for 5-HT (K_i = 11 nM). and the cys → glu mutant exhibiting a 3.4-fold increase in affinity for 5-HT (K_i = 86 nM).

To determine whether other agonists would display a similar increase in affinity for the mutant receptors, two known agonists (DOM and DOB) were tested with both the native and cys → lys mutant. Figure 8 shows the radioligand binding data of ^{3}H-ketanserin labeled native and mutant 5-HT_{2A} receptors in the

presence of agonists and antagonists. 0.5 nM ^{3}H-ketanserin was used to label the native and mutant 5-HT_{2A} receptors expressed in COS-7 cells. The DOM and DOB agonists show increased affinity for the mutant receptor, as is seen for 5-HT. The K_i for DOM shows a 5-fold increase, while the K_i for DOB shows a 7.4-fold increase.

To determine if the mutant 5-HT_{2A} receptors would exhibit an increase in agonist potency relative to the native 5-HT_{2A} receptor, 5-HT stimulation of the native and mutant 5-HT_{2A} receptors was measured using an IP production assay. Figure 9 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT_{2A} receptors. Both the cys → lys and cys → glu mutant receptor curves exhibit a leftward shift away from the native curve in the 5-HT dose-response indicating that there was an increase in 5-HT potency at the mutant receptors. The cys → lys and cys → glu mutant receptors displayed EC_{50} values of 25 nM and 61 nM, respectively, as compared to the native 5-HT_{2A} receptor which had an EC_{50} value of 152 nM.

Figure 10 shows the basal activity and 5-HT stimulation of the native and mutant 5-HT_{2A} receptors. As can be seen, both the cys → lys and the cys → glu mutant 5-HT_{2A} receptors show dramatic increases in basal intracellular inositol phosphate (IP) accumulation compared to the native receptor. The cys → lys mutant receptor produced a 345% (8-fold) increase in IP levels over the vector control. The cys → glu mutant receptor produced a 158% (3.7-fold) increase in IP levels over the vector control. Upon the addition of 10 μM 5-HT, both the native and mutant receptors produced an additional increase in IP production. The basal activity of the cys → lys mutant was 48% of that of the maximally stimulated native 5-HT_{2A} receptor. The basal activity of the cys → glu mutant was 31% of that of the maximally stimulated native 5-HT_{2A} receptor.

In order to determine whether the above results were due to an increase in the number of expressed mutant receptors rather than to a change in the properties of the mutated receptors, saturation curves were generated. Figure 11 shows a saturation analysis of ^{3}H-ketanserin labeled native and cys → lys mutant receptors. B_{MAX} values were determined by a BCA assay. For the native receptor the B_{MAX} = 193 +/- 37 fmol/mg, while for the cys → lys mutant receptor, the

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B_{MAX} = 218 +/- 31 fmol/mg. There is no significant difference in the B_{MAX} values for the native and mutant receptors. The K_D of ^{3}H-ketanserin also did not differ between the native and mutant receptors. These data demonstrate that the results were not due to an increase in number of expressed mutant receptors compared to expressed native receptors.

Thus, the mutated 5-HT_{2A} receptors meet all the criteria for constitutively activated receptors; they show a higher affinity for agonists; they show a higher potency for 5-HT; and they show activation (coupling) of the G protein second messenger pathway (IP production) even in the absence of agonist.

Example 2: Constitutive Activation of 5-HT_{2C} Receptor

Materials and Methods For Site-directed Mutagenesis:

The rat 5-HT_{2C} receptor cDNA was ligated into the mammalian expression vector pcDNA3 (Invitrogen) using BamHI (Gibco). This construct served as the native template for site-directed mutagenesis performed using Clonetech's Transformer kit. Mutagenic primers (Midland Certified Reagent Company) were designed complementary to amino acids #308-317 of the native 5-HT_{2C} cDNA, while changing amino acid #312 from serine (TCC) to lysine (AAG) or phenylalanine (TTC). The same primers were designed to incorporate an Sca1 restriction site at amino acid #314 by changing the third codon in valine from GTC to GTA. The selection primer, complementary to bases 2081-3017 of the pcDNA3 vector, was designed to remove a unique Sma1 site by changing glycine at base 2093 from GGG to GGA. Phosphorylated primers were annealed to 10ng of alkaline denatured plasmid template by heating to 65°C for 5 minutes and cooling slowly to 37°C. Mutant DNA was synthesized using T4 DNA polymerase and ligase (Clonetech) by incubating for 1 hour at 37°C, followed by digestion with Sma1 (Gibco) and transformation of BMH71-18mutS E. coli (Clonetech). Plasmid was purified using the Wizard miniprep kit (Promega), digested with Sma1, and used to transform DH5α E. coli (Gibco). Individual colonies were isolated and plasmid DNA was digested with Sca1 to screen for S312K and S312F mutants (Gibco). S312K and S312F mutant plasmids contain an additional Sca1 site and appear as two bands (2.3Kb and 7.6Kb) when run on a 1% agarose gel. DNA sequencing (Sequenase version 2.1 kit USB, ^{35}Sd-ATP NEN)

was performed to confirm the incorporation of lysine or phenylalanine at amino acid #312. Sequencing reactions were run on a 5% acrylamide/bis (19:1) gel (BioRad) for 2 hours at 50°C, dried for 2 hours at 80°C, and exposed to Kodak Biomax MR film for 24 hours at -80°C.

In Figure 31 is shown the amino acid sequence of the 5-HT_{2c} ser → lys mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 32 shows the resulting DNA sequence of the 5-HT_{2c} ser → lys mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 lysine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT_{2c} ser → lys mutant receptor, Figure 33 shows the base, which was mutated to create the Sca1 site, as a larger outlined letter indicated with an arrow.

In Figure 34 is shown the amino acid sequence of the 5-HT_{2c} ser → phe mutant receptor with the mutated amino acid shown as a larger outlined letter. Figure 35 shows the resulting DNA sequence of the 5-HT_{2c} ser → phe mutant receptor including the 5′ and 3′ untranslated regions with the translated codons underlined. The bases specifying the #312 phenylalanine mutant are shown as larger outlined letters, and the starting and ending locations of the primer are also indicated. In addition to showing the mutated DNA sequence of the 5-HT_{2c} ser → phe mutant receptor, Figure 36 shows the base, which was mutated to create the Sca1 site, as a larger outlined letter indicated with an arrow.

Cell culture and transfection:

COS-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 10% fetal bovine serum (Sigma) in 5% CO_2 at 37°C and subcultured 1:8 twice a week. Twenty-four hours prior to transfection, cells were seeded at 30% confluence in 100mm dishes for radioligand binding assays or at 10^5 cells/well in 24 well cluster plates for PI assays. Cells were transfected with native or mutant 5-$HT2_c$ cDNA using Lipofectamine (Gibco). This was accomplished by combining 20 μl of lipofectamine with 2.5 μg plasmid per 100mm dish or 2 μl lipofectamine and 0.25 μg plasmid per well. Transfections were performed in serum-free DMEM for 4 hours at 37°C.

Radioligand binding:

Thirty-six hours after transfection, membranes were prepared from COS-7 cells by scraping and homogenizing in 50mM Tris-HCl / 5mM $MgCl_2$ / 0.5mM EDTA pH 7.4 (assay buffer) and centrifugation at 10,000xg for 30 minutes. Membranes were resuspended in assay buffer, homogenized and centrifuged again. Following resuspension in assay buffer, 1 ml membrane aliquots (approximately 10 μg protein measured by BCA assay) were added to each tube containing 1ml of assay buffer with 1nM ^{3}H-mesulergine and competing drugs. 10μM mianserin was used to define non-specific binding. Saturation experiments were performed using ^{3}H-mesulergine (0.1nM-5.0nM) or ^{3}H-5-HT (0.1nM-30nM) in the absence of presence of 10μM GppNHp (RBI). Samples were incubated at 37°C for 30 minutes, filtered on a Brandel cell harvester, and counted in Ecoscint cocktail (National Diagnostics) in a Beckman liquid scintillation counter at 40% efficiency.

Phosphatidylinositol hydrolysis:

Inositol phosphate (IP) production was measured using a modified combination of the methods of Berridge et al., 1982 and Conn and Sanders-Bush 1985. Briefly, 24 hours after transfection, cells were washed with PBS and labeled with 0.25μCi/well of ^{3}H-myoinositol (NEN) in inositol-free/serum-free DMEM (Gibco) for 12 hours at 37°C. Following labeling, cells were washed with PBS and preincubated in inositol-free/serum-free DMEM with 10mM LiCl and 10μM pargyline (assay medium) for 10 minutes at 37°C. When antagonists were used they were added during the 10 minute preincubation period. 5-HT (Sigma), or assay medium alone, was added to each well and incubation continued for an additional 35 minutes (Westphal et al., 1995). Assay medium was removed and cells were lysed in 250 μl of stop solution (1M KOH / 18mM NaBorate / 3.8mM EDTA) and neutralized by adding 250μl of 7.5% HCl. The contents of each well were extracted with 3 volumes of chloroform:methanol (1:2), centrifuged 5 minutes at 10,000xg, and the upper layer loaded onto a 1ml AG1-X8 resin (100-200 mesh, BioRad) column. Columns were washed with 10mls of 5mM myoinositol and 10mls of 5mM NaBorate / 60mM NaFormate. Total IPs were eluted with 3mls of 0.1M formic acid / 1M ammonium formate. Radioactivity was

measured by liquid scintillation counting in Ecoscint cocktail.

Stable Transfection:

Although not yet fully characterized, it has been found possible to create a stable cell line expressing mutant receptors by the following method. The rat 5-HT_{2C} cDNA (edited VSI isoform) was used as a template for site-directed mutagenesis to convert amino acid 312 from serine to lysine as previously described. Native and S312K 5-HT_{2C} cDNAs were ligated into the BamHI/EcoRI site of the pZeoSV2+ mammalian expression vector (Invitrogen) containing the zeocin resistance gene. NIH3T3 cells (ATCC) were stably transfected using the high efficiency BES method. Briefly, cells were seeded at $5x10^5$ cells/100mm culture dish in complete medium (DMEM/10%FBS) and grown in 5% CO_2 at 37° overnight. Twenty micrograms of pZeoSV2/5-HT_{2C} DNA (linearized with BglII) was mixed with 500µl of 0.25M $CaCl_2$ and 500µl of 2x BES solution (50mM N,N-bis-2-hydroxyethyl-2-aminoethanesulfonic acid; 280mM NaCl; 1.5mM Na_2HPO_4; pH to *6.95)* and incubated at 25°C for 20 minutes. The solution was added dropwise on top of the cells. The cells were incubated for 20 hours at 35°C in 3% CO_2, washed twice with PBS, complete medium replenished, and incubated for 48 hours at 37°C in 5% CO_2. Cells were split 1:4 into complete medium containing 500µg/ml zeocin. Individual colonies were isolated and tested for 5-HT_{2C} receptor expression by ^{3}H-mesulergine binding.

Demonstration of Constitutive Activation:

Constitutive activity of the mutated 5-HT_{2C} receptors is demonstrated by the fact that the mutated receptors also exhibit all the hallmark characteristics established for constitutive activation: a showing of increased agonist affinity, increased agonist potency, and coupling to the G protein second messenger system in the absence of agonist.

Figure 12 shows the competition curves of 5-HT for ^{3}H-mesulergine labeled native and mutant 5-HT_{2C} receptors. 0.5nM ^{3}H-mesulergine was used to label the native and mutant receptors transiently transfected in COS-7 cells. As shown in Figure 12, the 5-HT competition isotherms for ^{3}H-mesulergine labeled ser → lys and ser → phe mutant receptors display a marked leftward shift compared with native receptors. The affinity of 5-HT for ser → lys mutant receptors increased

almost 30-fold from 203 nM in the native to 6.6 nM in the ser → lys mutant. Similarly, but on a smaller scale, the ser → phe mutation resulted in a 3-fold increase in 5-HT affinity to 76 nM.

To determine whether other agonists would display a similar increase in affinity for the mutant receptors, two known agonists, 5-methoxytryptamine and DOB were tested with the ser → lys mutant. Figure 13 shows the radioligand binding analysis of native and mutant 5-HT_{2C} receptors in the presence of agonists and antagonists. Native and mutant 5-HT_{2C} receptors expressed in COS-7 cells were labeled with 1 nM 3H-mesulergine. The 5-MT and DOB agonists show increased affinity for the mutant receptor, as is seen for 5-HT. 5-methoxy-tryptamine and DOB display an 89-fold and 38-fold increase, respectively, in affinity for the ser → lys mutant receptors.

To determine if the mutant 5-HT_{2C} receptors would exhibit an increase in agonist potency relative to the native 5-HT_{2C} receptor, 5-HT stimulation of the native and mutant 5-HT_{2C} receptors was measured using an IP production assay. Figure 14 shows the stimulation of IP production in COS-7 cells expressing native or mutant 5-HT_{2C} receptors. Both the ser → lys and ser → phe mutant receptor curves exhibit a leftward shift away from the native curve in the 5-HT dose-response indicating that there was an increase in 5-HT potency for the mutant receptors. The shifts were similar in magnitude to the shifts in the 5-HT competition binding isotherms. Figure 15 shows the 5-HT stimulation of IP production in COS-7 cells transfected with the ser → lys or ser → phe mutated receptors. As shown in Figure 15, the EC_{50} value for 5-HT mediated stimulation of IP production increased from 70 nM in cells transfected with native receptors to 2.7 nM in the ser → lys mutant and 28 nM in the ser → phe mutant.

Figure 16 shows the effect of the ser → lys and ser → phe mutations on basal levels of IP production by the mutated 5-HT_{2C} receptors. Cells transfected with native 5-HT_{2C} receptors displayed a small increase (9%, 225dpm) in basal IP production over cells transfected with vector alone. Transfection with ser → lys and ser → phe mutant 5-HT_{2C} receptors resulted in 5-fold and 2-fold increases, respectively, in basal levels of IP production when compared with cells expressing native 5-HT_{2C} receptors. Basal levels of IP stimulated by ser → lys mutant

receptors represented 50% of total IP production stimulated by native receptors in the presence of 10 μM 5-HT. 5-HT stimulated IP production 10 fold over basal levels in cells transfected with native receptors and 2-fold over basal levels in cells transfected with ser → lys mutant receptors. However, 5-HT elicited the same maximal IP response in cells transfected with native or mutant receptors.

Since receptor density can influence agonist binding affinity and potency in stimulating second messenger systems, saturation curves were generated. Therefore, ^{3}H-mesulergine saturation analyses and Scatchard transformations were performed in parallel to control for variations in transfection efficiency and receptor expression levels. As shown in Figure 15, the 5-HT_{2C} receptor density was greater in cells transfected with native receptors than in cells transfected with either the ser → lys or the ser → phe mutant receptors. These data indicate that the increase in agonist binding affinity and potency of the mutated receptors did not result from increased receptor expression, but directly resulted from the mutations.

Thus, like the mutated 5-HT_{2A} receptors, the mutated 5-HT_{2C} receptors meet all the criteria for constitutively activated receptors; they show a higher affinity for agonists; they show a higher potency for 5-HT; and they show activation (coupling) of the G protein second messenger pathway (IP production) even in the absence of agonist.

Inverse Agonism at Constitutively Activated Serotonin Receptors

As noted above, the discovery and elucidation of the mechanisms of action of constitutively activated receptors has led to the recognition of a new class of receptor antagonists, identified as inverse agonists. The mutated 5-HT_{2A} and 5-HT_{2C} receptors of this invention were used to test the activity of known serotonin receptor antagonists. Figure 8 shows the binding affinities of four known 5-HT_{2A} antagonists to the native and cys → lys mutant 5-HT_{2A} receptors. There is an apparent decease in the binding affinity of methysergide and mianserin at the mutant 5-HT_{2A} receptors, but no change in binding affinity for spiperone and ketanserin. However, as shown in Figure 17, both spiperone and ketanserin reversed the constitutive stimulation of IP production in cells expressing the mutant 5-HT_{2A} receptor. Ketanserin and spiperone decreased the constitutive IP

stimulation by 80% and 58% respectively.

Several antipsychotic drugs presently in use are thought to act at the 5-HT_{2A} receptor. As shown in Figure 18, all these drugs, chlorpromazine, haloperidol, loxapine, clozapine, and risperidone as well as spiperone reduce the constitutively activated IP basal activity of the mutated 5-HT_{2A} receptor.

The constitutively active ser → lys mutated 5-HT_{2C} receptor of this invention can also be used to screen compounds for inverse agonist activity. Figure 19 shows that two classical 5-HT_{2C} receptor antagonists, mianserin and mesulergine, exhibit inverse agonist activity by decreasing basal levels of PI hydrolysis associated with the constitutively active 5-HT_{2C} mutant receptor. The inverse agonism of these compounds is apparent both in the presence and absence of serotonin.

The demonstration of inverse agonism at the mutated 5-HT_{2A} and 5-HT_{2C} receptors further characterizes the mutated serotonin receptors of this invention as being constitutively active. Not only have the 5-HT_{2A} and 5-HT_{2C} receptors been mutated to a constitutively active form, but a method has been disclosed for mutating all mammalian G protein-coupled monoamine receptors, including serotonin receptors, to a constitutively active form. Unlike the case of the M5 muscarinic acetylcholine receptor where mutations in the third cytoplasmic loop do not produce constitutive activation, the present invention clearly demonstrates that mutations in the third cytoplasmic loop of G protein-coupled serotonin receptors may be used to induce constitutive activation. Previously, third intracellular loop mutations near the transmembrane region had only been found to produce constitutively active receptors of the adrenergic type. With the present discoveries, it is now recognized that the alignment and positional mutation method of this invention is applicable to the general class of monoamine receptors of which the adrenergic and serotonin receptors are major subclasses. Further, based upon the present discoveries, it is expected that mutations may be introduced at other sites in the third cytoplasmic loop which will constitutively activate the G protein-coupled monoamine receptors including the serotonin receptors.

Additional Advances Enabled By The Discoveries Of The Present Invention:

Figures 20A and 20B show the DNA and amino acid sequences for the human 5-HT_{2A} receptors. In Figure 20A, it can be seen that the sixth transmembrane domain has the same WxPFFI conserved sequence (outlined type) as seen in the rat receptors. Figures 21A and 21B show the DNA and amino acid sequences for the human 5-HT_{2C} receptors. In Figure 21A it can be seen that the sixth transmembrane domain also has the same WxPFFI conserved sequence (outlined type) as seen in the rat receptors. Both of these human receptors may, therefore, be similarly aligned with the rat α1-adrenergic, 5-HT_{2A}, and 5-HT_{2C} receptors to identify the amino acid positions which may be mutated to produce constitutively active human receptors following the methodologies of this invention.

Having identified mutations which constitutively activate the 5-HT_{2A} and 5-HT_{2C} serotonin receptors, it is now possible to create transgenic mammals incorporating these mutations using techniques well known in the art. This will provide an opportunity to study the physiological consequences of constitutive receptor activation and may lead to the development of novel therapeutic agents.

Those skilled in the art will recognize that various modifications, additions, substitutions and variations of the illustrative examples set forth herein can be made without departing from the spirit of the invention and are, therefore, considered within the scope of the invention.

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2. Kjelsberg, M., Cotecchia, S., Ostrowski, J. Caron, M. and Lefkowitz, R. (1992) *Constitutive Activation of the α_{1B}-Adrenergic Receptor by All Amino Acid Substitutions at a Single Site*. J. Biol. Chem. Vol. 267, 1430-1433

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5. The native rat 5-HT_{2A} receptor cDNA was generously donated by Dr. David Julius of the University of California, San Francisco.

6. The native rat $5HT_{2C}$ receptor cDNA was generously donated by Dr. Beth Hoffman of the National Institutes of Health.

CLAIMS

What is claimed is:

1. A method of constitutively activating targeted G protein-coupled mammalian monoamine receptors comprising the following steps:

a. aligning a conserved amino acid sequence occurring in the sixth transmembrane domain of the targeted monoamine receptor with the conserved amino acid sequence in the sixth transmembrane domain of a second monoamine receptor for which a constitutively activated form having a mutation in the third intracellular loop is known;

b. identifying in the aligned receptor sequences the amino acid position in the targeted monoamine receptor which corresponds to the amino acid position in the third intracellular loop which produced constitutive activation in the second monoamine receptor; and

c. mutating, by site-directed mutagenesis, the identified amino acid position in the targeted monoamine receptor so that a different amino acid is substituted for the amino acid occurring in the native targeted receptor.

2. The method of claim 1 in which the targeted monoamine receptor is a G protein-coupled serotonin receptor.

3. The method of claim 2 in which the G protein-coupled serotonin receptor is the 5-HT_{2A} receptor.

4. The method of claim 2 in which the G protein-coupled serotonin receptor is the 5-HT_{2C} receptor.

5. The method of claim 1 in which the conserved amino acid sequence within the sixth transmembrane domain used for the alignment is WxPFFI, where x represents that any amino acid may occur at that position.

6. A method of constitutively activating G protein-coupled mammalian serotonin receptors comprising the following steps:

a. aligning a conserved amino acid sequence occurring in the sixth transmembrane domain of the serotonin receptor with the conserved amino acid sequence in the sixth transmembrane domain of the α_{1B}-adrenergic receptor for which a constitutively activated form having

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a mutation in the third intracellular loop is known;

b. identifying in the aligned receptor sequences the amino acid position in the serotonin receptor which corresponds to the amino acid position in the third intracellular loop which produced constitutive activation in the α_{1B}-adrenergic receptor; and

c. mutating, by site-directed mutagenesis, the identified amino acid position in the serotonin receptor so that a different amino acid is substituted for the amino acid occurring in the native serotonin receptor.

7. The method of claim 6 in which the G protein-coupled serotonin receptor is the 5-HT_{2A} receptor.

8. The method of claim 6 in which the G protein-coupled serotonin receptor is the 5-HT_{2C} receptor.

9. The method of claim 6 in which the conserved amino acid sequence within the sixth transmembrane domain used for the alignment is WxPFFI, where x represents that any amino acid may occur at that position.

10. The constitutively active 5-HT_{2A} receptor in which the amino acid at position number 322 has been mutated from the cysteine found in the native receptor to an amino acid selected from the group consisting of lysine, glutamic acid, and arginine.

11. The constitutively active 5-HT_{2C} receptor in which the amino acid at position number 312 has been mutated from the serine found in the native receptor to an amino acid selected from the group consisting of lysine and phenylalanine.

12. The DNA encoding the constitutively active 5-HT_{2A} receptor in which the amino acid at position number 322 has been mutated from the cysteine found in the native receptor to an amino acid selected from the group consisting of lysine, glutamic acid, and arginine.

13. The DNA encoding the constitutively active 5-HT_{2C} receptor in which the amino acid at position number 312 has been mutated from the serine found in the native receptor to an amino acid selected from the group consisting of lysine and phenylalanine.

14. A method of efficiently minimizing the number of full DNA sequencings, which must be performed on the colonies resulting from site-directed mutagenesis employing vectors, by eliminating most colonies not containing the desired mutation and by tagging colonies containing the desired mutation for easy identification comprising the following steps:

a. creating two primers, the first of which will remove a restriction site occurring in the original form of the vector and the second of which will introduce the desired mutation as well as a second mutation which specifies a unique restriction site not found in the primer;

b. annealing the primers to the vector;

c. synthesizing the second strands;

d. exposing the double stranded DNA to the restriction enzyme for the restriction site which occurs on the original vector thereby digesting the DNA containing the restriction site so that it cannot be taken up during a subsequent transformation;

e. transforming the test organism with the remaining double stranded circular DNA; and

f. testing the resulting colonies to see if they contain DNA which can be digested by the restriction enzyme for the unique site introduced by the second primer

whereby only DNA from those colonies which have incorporated the desired mutation will be digested with the restriction enzyme for the unique restriction site and the presence of such digestion indicates that that colony contains the desired mutation.

15. The method of claim 14 in which the following additional steps are performed after step e and before step f of claim 14:

e'. repeating a restriction digest using the restriction enzyme for the restriction site which occurs on the original vector; and

e''. tansforming the test organism with the remaing double stranded circular DNA.

16. The constitutively active 5-HT_{2A} receptor coded by the DNA sequence specified in Figure 24 which DNA also contains a mutation creating a unique

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restriction site.

17. The constitutively active 5-HT_{2A} receptor coded by the DNA sequence specified in Figure 27 which DNA also contains a mutation creating a unique restriction site.

18. The constitutively active 5-HT_{2A} receptor coded by the DNA sequence specified in Figure 30 which DNA also contains a mutation creating a unique restriction site.

19. The constitutively active 5-HT_{2C} receptor coded by the DNA sequence specified in Figure 33 which DNA also contains a mutation creating a unique restriction site.

20. The constitutively active 5-HT_{2C} receptor coded by the DNA sequence specified in Figure 36 which DNA also contains a mutation creating a unique restriction site.

21. The use of the constitutively activated mammalian G protein-coupled monamine receptor to screen for agonists, inverse agonists, and antagonists not previously identified as such at the native receptor.

22. The method of claim 21 where the mammalian G protein-coupled monoamine receptor is a serotonin receptor.

23. A transgenic mammal having incorporated and expressed in its genome a constitutively activated monoamine G protein-coupled receptor.

24. The transgenic mammal of claim 23 wherein the constitutively activated monoamine G protein-coupled receptor is a serotonin receptor.

25. The method of constitutively activating G protein-coupled receptors as described and illustrated in the specification.

26. The method of efficiently minimizing the number of full DNA sequencings as described and illustrated in the specification.

27. The constitutively activated receptors as described and illustrated in the specification.

28. DNA encoding constitutively activated receptors as described and illustrated in the specification.

29. The invention as described and illustrated in the specification.

Rat 5-HT_{2A}

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

1021 cgtgcaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 1A

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 1A - CONTINUED

Rat 5-HT_{2A}

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKACKVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 1B

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Rat 5-HT_{2c}

ORIGIN 23 bp upstream of HindIII site.

1 ggcgctctgg tgctcactga ggaagcttcc ttaggtgtac cgatcttaat gattgagccc

61 ttggagcagc aagattgtta atcttggttg ctcctttggc ctgtctatcc cttaccttcc

121 tattacatat gaacttttct tcgttctgca catcgattgt cgtcggcgtc gtggagatcg

181 tcgtggtgct ccggtggtgg tcttcgtccg cttagaatag tgtagttagt taggggcctt

241 caaagaagaa agaagaagcg attggcgcgg agagatgctg gaggtgtcag tttctatgct

301 agagtagggt agtgaaacaa tccccagcca aacctttccg gggggcgcag gttgcccaca

361 ggaggtcgac ttgccggcgc tgtccttcgc gccgagctcc ctccatcctt ctttccgtct

421 gctgagacgc aaggttgcgg cgcgcacgct gagcagcgca ctgactgccg cgggctccgc

481 tgggcgattg cagccgagtc cgtttctcgt ctagctgccg ccgcggcgac ctgcctggtc

541 ttcctcccgg acgctagcgg gttgtcaact attacctgca agcataggcc aacgaacacc

601 ttctttccaa attaattgga atgaaacaat tctgttaact tcctaattct cagtttgaaa

661 ctctggttgc ttaagcctga agcaatcatg gtgaaccttg gcaacgcggt gcgctcgctc

721 ctgatgcacc taatcggcct attggtttgg caattcgata tttccataag tccagtagca

781 gctatagtaa ctgacacttt taattcctcc gatggtggac gcttgtttca attcccggac

841 ggggtacaaa actggccagc actttcaatc gtcgtgatta taatcatgac aatagggggc

901 aacattcttg ttatcatggc agtaagcatg gagaagaaac tgcacaatgc aaccaattac

961 ttcttaatgt ccctagccat tgctgatatg ctggtgggac tacttgtcat gcccctgtcc

1021 ctgcttgcta ttctttatga ttatgtctgg cctttaccta gatatttgtg ccccgtctgg

1081 atttcactag atgtgctatt ttcaactgcg tccatcatgc acctctgcgc catatcgctg

1141 gaccggtatg tagcaatacg taatcctatt gagcatagcc ggttcaattc gcggactaag

1201 gccatcatga agattgccat cgtttgggca atatcaatag gagtttcagt tcctatccct

FIGURE 2A

1261 gtgattggac tgagggacga aagcaaagtg ttcgtgaata acaccacgtg cgtgctcaat

1321 gaccccaact tcgttctcat cgggtccttc gtggcattct tcatcccgtt gacgattatg

1381 gtgatcacct acttcttaac gatctacgtc ctgcgccgtc aaactctgat gttacttcga

1441 ggtcacaccg aggaggaact ggctaatatg agcctgaact ttctgaactg ctgctgcaag

1501 aagaatggtg gtgaggaaga gaacgctccg aaccctaatc cagatcagaa accacgtcga

1561 aagaagaaag aaaagcgtcc cagaggcacc atgcaagcta tcaacaacga aaagaaagct

1621 tccaaagtcc ttggcattgt attctttgtg tttctgatca tgtggtgccc gtttttcatc

1681 accaatatcc tgtcggttct ttgtgggaag gcctgtaacc aaaagctaat ggagaagctt

1741 ctcaatgtgt ttgtgtggat tggctatgtg tgttcaggca tcaatcctct ggtgtacact

1801 ctctttaata aaatttaccg aagggctttc tctaaatatt tgcgctgcga ttataagcca

1861 gacaaaaagc ctcctgttcg acagattcct agggttgctg ccactgcttt gtctgggagg

1921 gagctcaatg ttaacattta tcggcatacc aatgaacgtg tggctaggaa agctaatgac

1981 cctgagcctg gcatagagat gcaggtggag aacttagagc tgccagtcaa cccctctaat

2041 gtggtcagcg agaggattag tagtgtgtaa gcgaagagca gcgcagactt cctacaggaa

2101 agttcctgta ggaaagtcct ccccaccccc cgtgattttc ctgtgaatca taactaatgt

2161 aaatattgct gtgtgacaag acagtgtttt tataaatagc tttgcaaccc tgtactttac

2221 atcatgcgtt aatagtgaga ttcggg

FIGURE 2A - CONTINUED

Rat 5-HT_{2c}

MVNLGNAVRSLLMHLIGLLVWQFDISISPVAAIVTDTFNSSDGG

RLFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADML

VGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNP

IEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLI

GSFVAFFIPLTIMVITYFLTIYVLRRQTLMLLRGHTEEELANMSLNFLNCCCKKNGGE

EENAPNPNPDQKPRRKKKEKRPRGTMQAINNEKKASKVLGIVFFVFLIMWCPFFITNI

LSVLCGKACNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSKYLRCDYKPD

KKPPVRQIPRVAATALSGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS

NVVSERISSV

FIGURE 2B

Rat α_{1B}-adrenergic

MNPDLDTGHNTSAPAHWGELKDDNFTGPNQTSSNSTLPQLDVTR

AISVGLVLGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLPFS

ATLEVLGYWVLLSFFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLVTRR

KAILALLSVWVLSTVISIGPLLGWKEPAPNDDKECGVTEEPFYALFSSLGSFYIPLAV

ILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNFHEDTLSSTKAKGHNPR

SSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFIALPLGSLFSTLKPPDAVFKVVF

WLGYFNSCLNPIIYPCSSKEFKRAFMRILGCQCRGGRRRRRRRRLGACAYTYRPWTRG

GSLERSQSRKDSLDDSGSCMSGTQRTLPSASPSPGYLGRGTQPPVELCAFPEWKPGAL

LSLPEPPGRRGRLDSGPLFTFKLLGDPESPGTEGDTSNGGCDTTTDLANGQPGFKSNM

PLAPGHF

FIGURE 3A

Rat α_{1B}-adrenergic

1 gggcggactt taaaatgaat cccgatctgg acaccggcca caacacatca gcacctgccc

61 actggggaga gttgaaagat gacaacttca ctggccccaa ccagacctcg agcaactcca

121 cactgcccca gctggacgtc accagggcca tctctgtggg cctggtgctg ggcgccttca

181 tcctctttgc catcgtgggc aacatcttgg tcatcctgtc ggtggcctgc aaccggcacc

241 tgcggacgcc caccaactac tttatcgtca acctggccat tgctgacctg ctgttgagtt

301 tcacagtact gcccttctcc gctaccctag aagtgcttgg ctactgggtg ctgttgagtt

361 tcttctgtga catctgggca gcggtagatg tcctgtgctg tacggcctcc atcctgagcc

421 tatgtgccat ctccattgac cgctacattg gggtgcgata ctctctgcag taccccacgc

481 tggtcacccg caggaaggcc atcttggcgc tcctcagtgt gtgggtcttg tccacggtca

541 tctccatcgg gcctctcctt ggatggaaag aacctgcgcc caatgatgac aaagaatgtg

601 gggtcaccga agaacccttc tacgccctct tttcctccct gggctccttc tacatcccgc

661 tcgcggtcat cctggtcatg tactgccggg tctacatcgt ggccaagagg accaccaaga

721 atctggaggc gggagtcatg aaggaaatgt ccaactccaa ggagctgacc ctgaggatcc

781 actccaagaa ctttcatgag gacaccctca gcagtaccaa ggccaagggc cacaacccca

841 ggagttccat agctgtcaaa ctttttaagt tctccaggga aaagaaagca gccaaaacct

901 tgggcattgt agtcggaatg ttcatcttat gttggctccc cttcttcatc gctctcccgc

961 ttggctccct gttctccacc ctaaagcccc cggacgccgt gttcaaggtg gtgttctggc

1021 tgggctactt caacagctgc ctcaatccca tcatctaccc gtgctccagc aaggagttca

1081 agcgcgcctt catgcgtatc cttgggtgcc agtgccgcgg tggccgccgc cgccgccgcc

1141 gtcgccgtct aggcgcgtgc gcttacacct accggccgtg gacccgcggc ggctcgctgg

1201 agagatcaca gtcgcggaag gactctctgg atgacagcgg cagctgcatg agcggcacgc

1261 agaggaccct gccctcggcg tcgcccagcc cgggctacct gggtcgagga acgcagccac

FIGURE 3B

1321 ccgtggagct gtgcgccttc cccgagtgga aacccggggc gctgctcagc ttgccagagc

1381 ctcctggccg ccgcggccgt ctcgactctg ggccactctt caccttcaag ctcctgggcg

1441 atcctgagag cccgggaacc gaaggcgaca ccagcaacgg gggctgcgac accacgaccg

1501 acctggccaa cgggcagccc ggcttcaaga gcaacatgcc cctggcgccc gggcacttt

1561 agggtccctt ttcatcctcc ccctcaacac actcacacat cggggtgggg gagaacacca

1621 tcgtaggggc gggagggcgc gtgggggggag tgtcagccct aggtagacac agggtcgcaa

1681 ggggacaagg ggggaggggg gcggggagag gggcagctgc ttttctggca ggggcatggg

1741 tgccaggtac agcgaagagc tgggctgagc atgctgagag cgtgggggggc ccccctagtg

1801 gttccgggac ttaagtctct ctctcttctc tctctgtata tacataaaat gagttcctct

1861 attcgtattt atctgtgggt acacgtgcgt gtgtctgttc ggtgtacgtg tgggctgcat

1921 gggtgtgagt gtgaggcctg cccgcacgcg cgtgccgggg cagagcgagt gcgcccccctg

1981 gtgacgtcca ggtgtgttgt ttgtctcttg actttgtacc tctcaagccc ctccctgttc

2041 tctagtcaat gctggcactt tgataggatc ggaaaacaag tcagatatta aagatcattt

2101 ctcctgtg

FIGURE 3B - CONTINUED

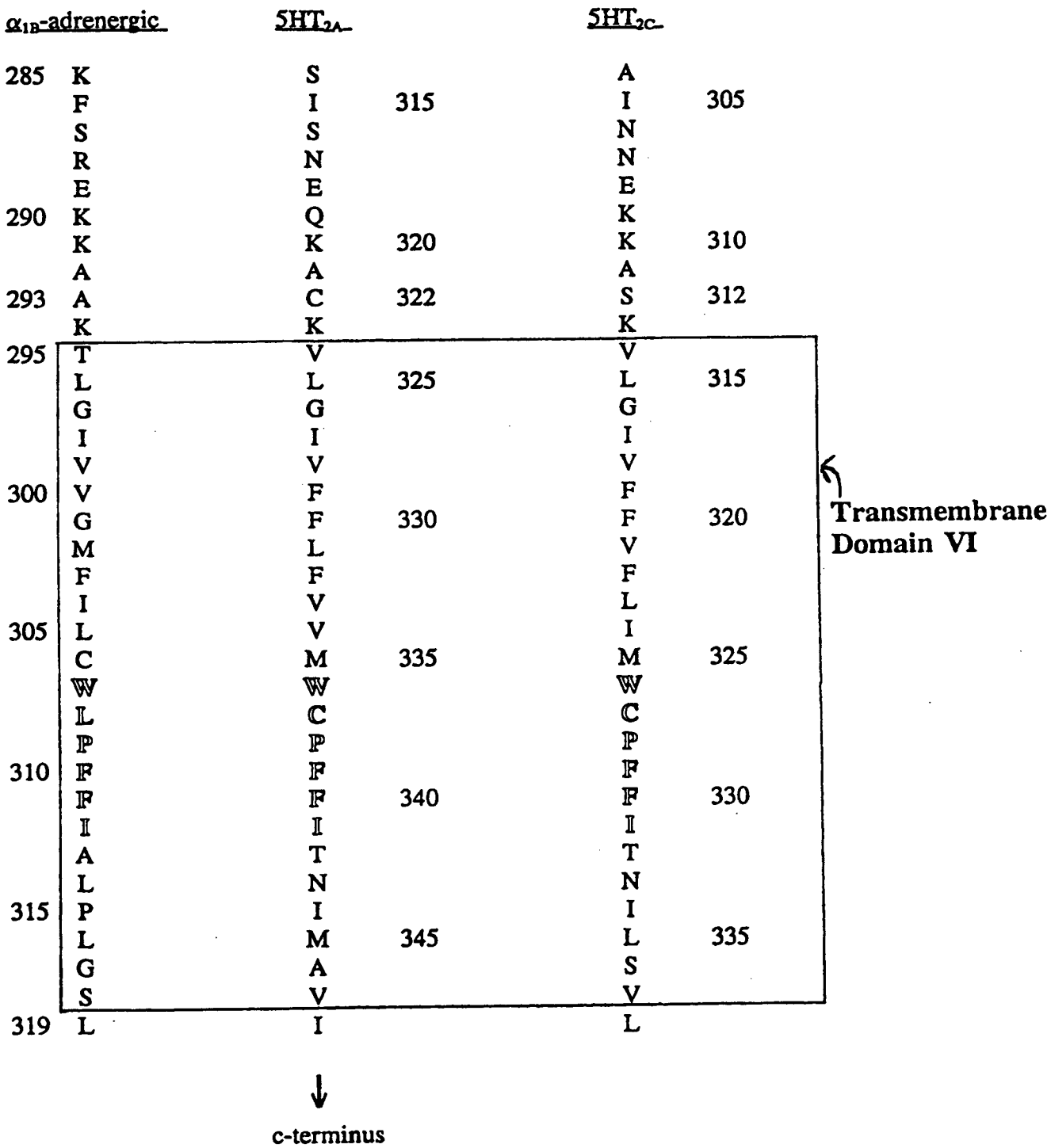

α1B-adrenergic
5HT2A
5HT2C
285 K F S R E K K A A K T L G I V V G M F I L C W L P F F I A L P L G S L
S I S N E Q K A C K V L G I V F F L V V M W C P F F I T N I M A V I
315
A I N N E K K A S K V L G I V F F V F L I M W C P F F I I T N I L S V L
305
290
320
310
293
322
312
295
325
315
300
330
320
305
335
325
310
340
330
315
345
335
319
Transmembrane Domain VI
c-terminus

FIGURE 4

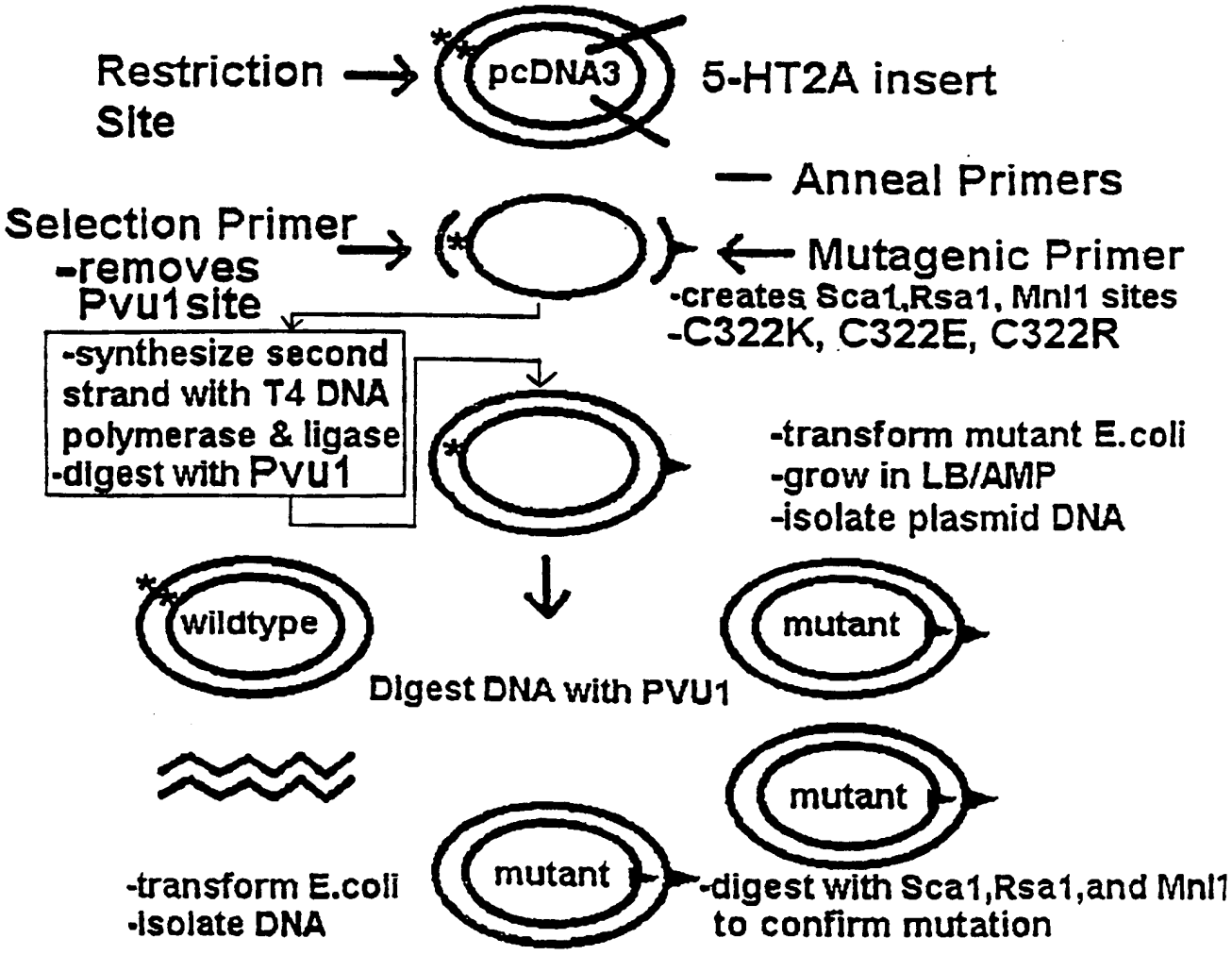


FIGURE 5

BNSDOCID: <WO__9838217A1_I_>

Site-directed mutagenesis procedure for the 5-HT2C receptor.

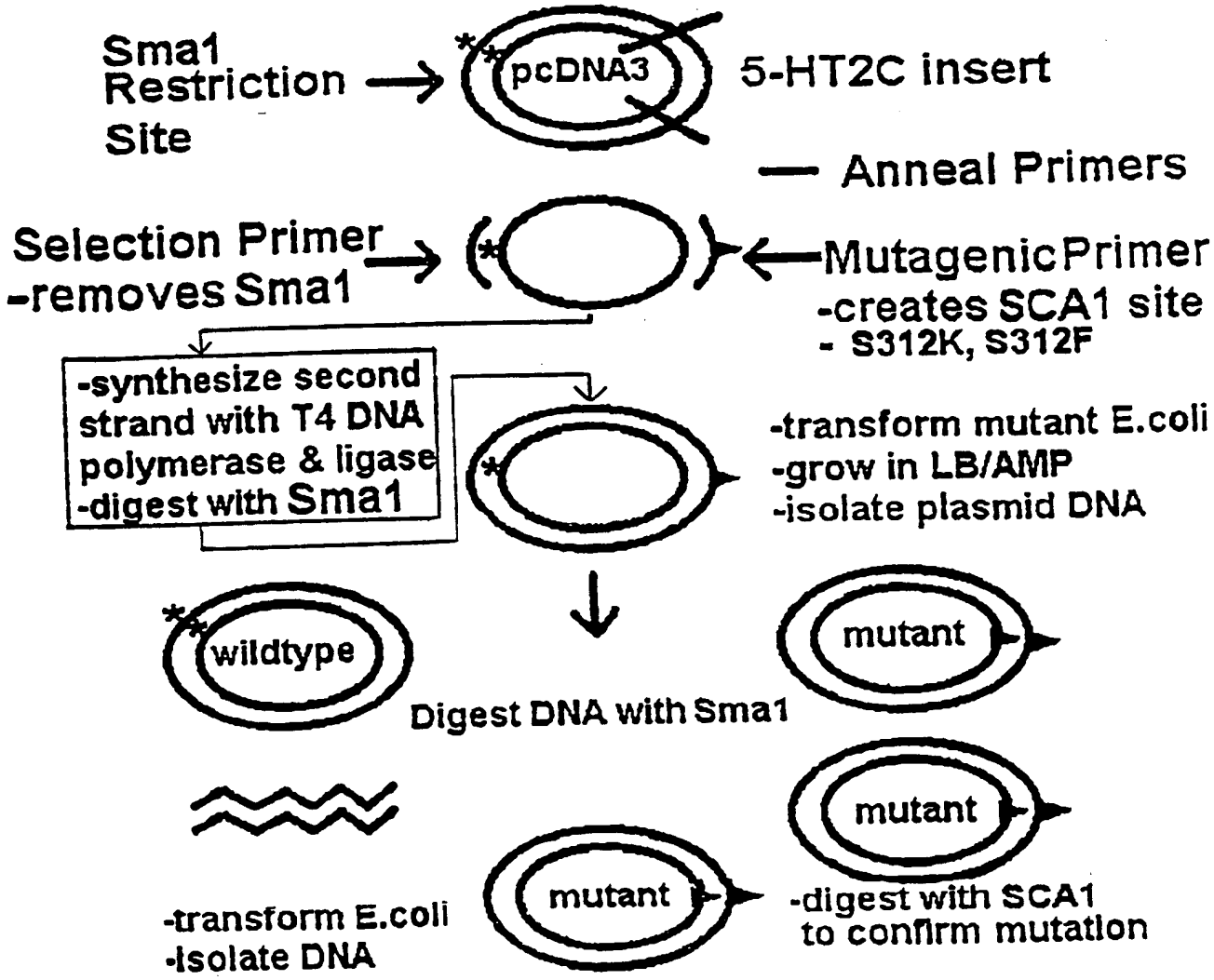


FIGURE 6

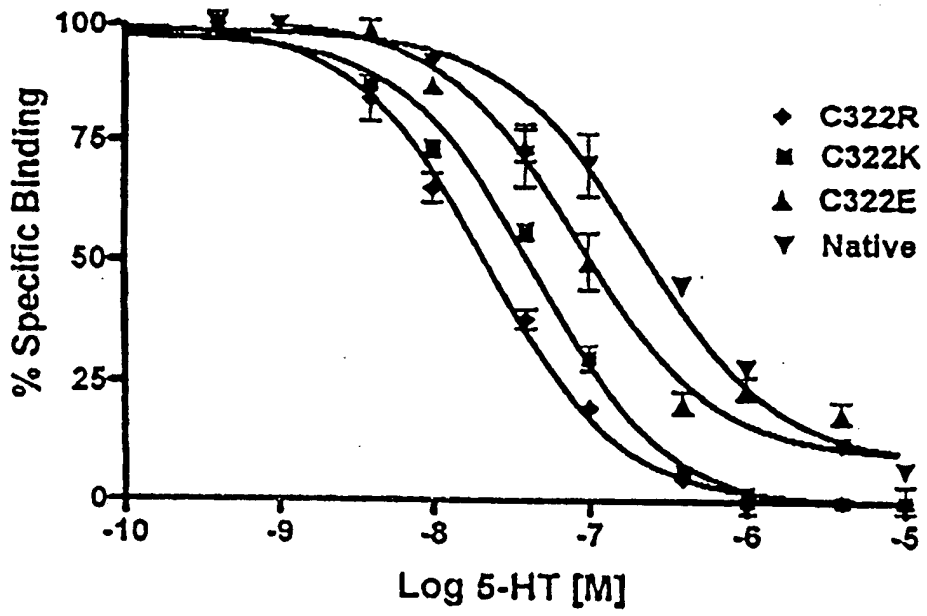


FIGURE 7

BNSDOCID: <WO 9838217A1 >

	Native 5-HT_{2A}	Cys→Lys Mutant	Cys→Arg Mutant	Cys→Glu Mutant
Agonists				
5-HT	293±3.0	25±2.1*	10±1.7	86±2.9
DOB	17±1.4	2.3±0.3*		
DOM	144±52	28±0.3*		
Antagonists				
Spiperone	1.1±0.1	2.4±1.0		
Methysergide	0.3±0.1	6.0±0.7*		
Ketanserin	1.0±0.3	1.0±0.1		
Mianserin	3.9±22	13±2.0*		

FIGURE 8

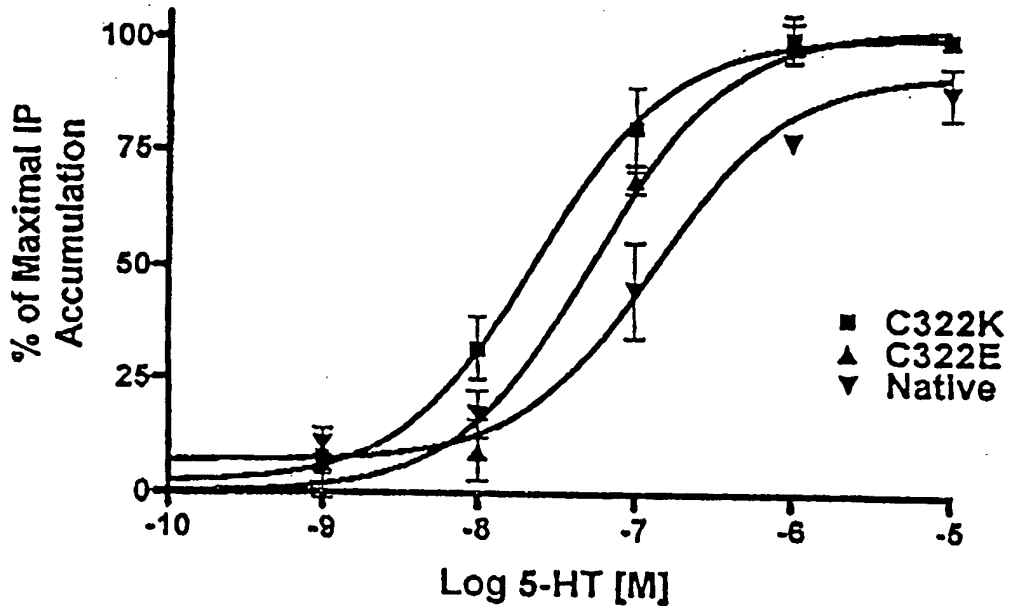


FIGURE 9

BNSDOCID: <WO 9838217A1 >

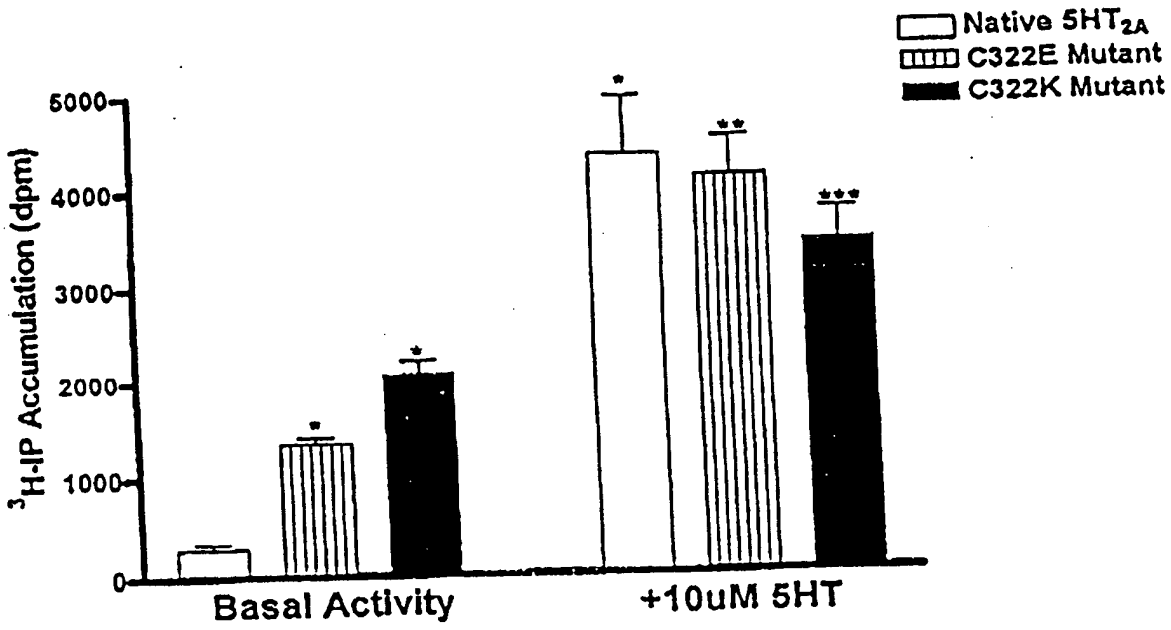
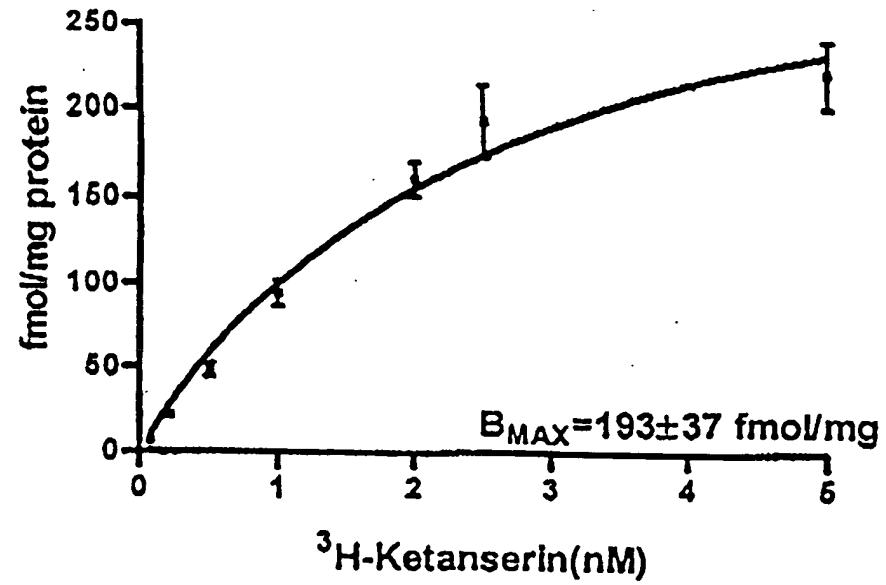

Native 5HT2A
C322E Mutant
C322K Mutant
3H-IP Accumulation (dpm)
5000
4000
3000
2000
1000
0
*
*
*
**
***
Basal Activity
+10uM 5HT

FIGURE 10



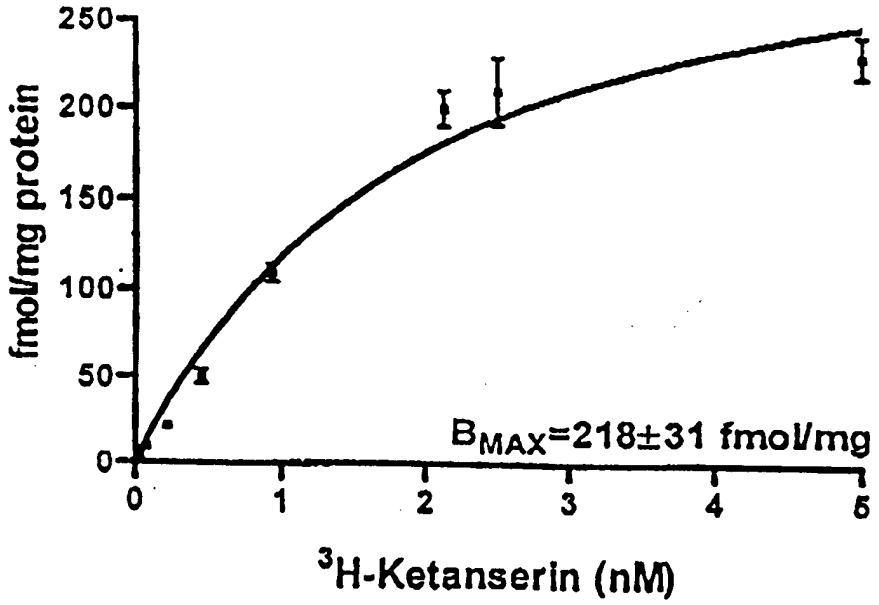


FIGURE 11

BNSDOCID: <WO 9838217A1 >

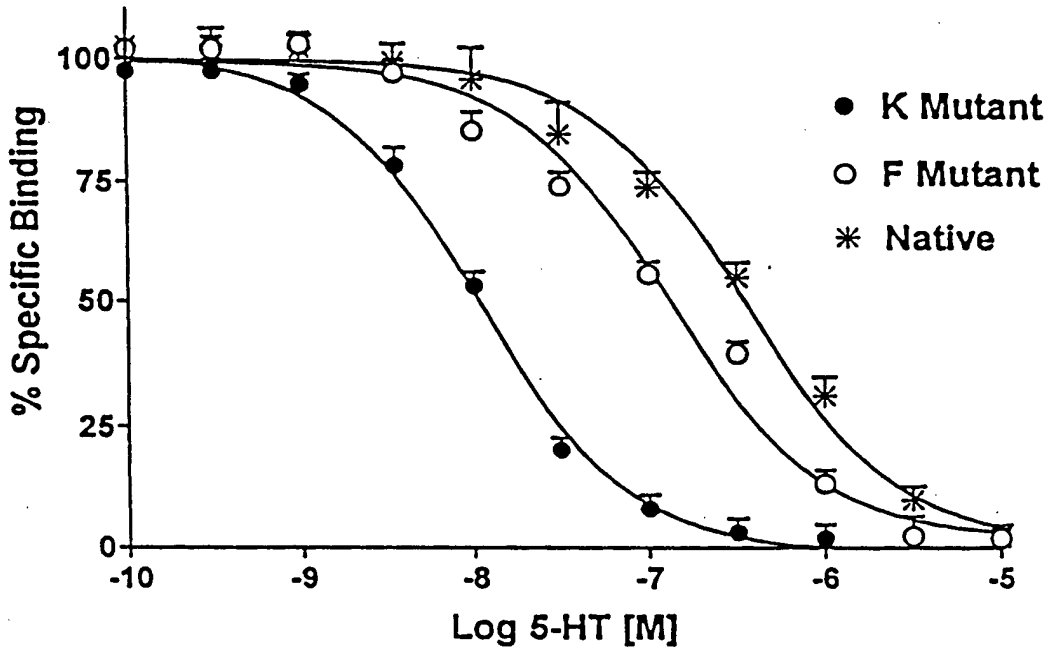

100
75
50
25
0
% Specific Binding
-10
-9
-8
-7
-6
-5
Log 5-HT [M]
K Mutant
F Mutant
Native

FIGURE 12

	Ki (nM)		
Agonists	Native	K Mutant	F Mutant
5-HT	203+/-10	6.6+/-1.2*	76+/-7.1*
5-MT	519+/-104	5.8+/-1.1*	ND
(+/-)DOB	256+/-38	6.7+/-0.7*	ND
Antagonists			
Mesulergine[a]	0.6+/-0.1	1.2+/-0.1*	1.3+/-0.2*
Mianserin	1.7+/-0.2	3.0+/-0.7**	ND
Methysergide	0.5+/-0.1	0.9+/-0.1**	ND

FIGURE 13

BNSDOCID: <WO_ 9838217A1 | >

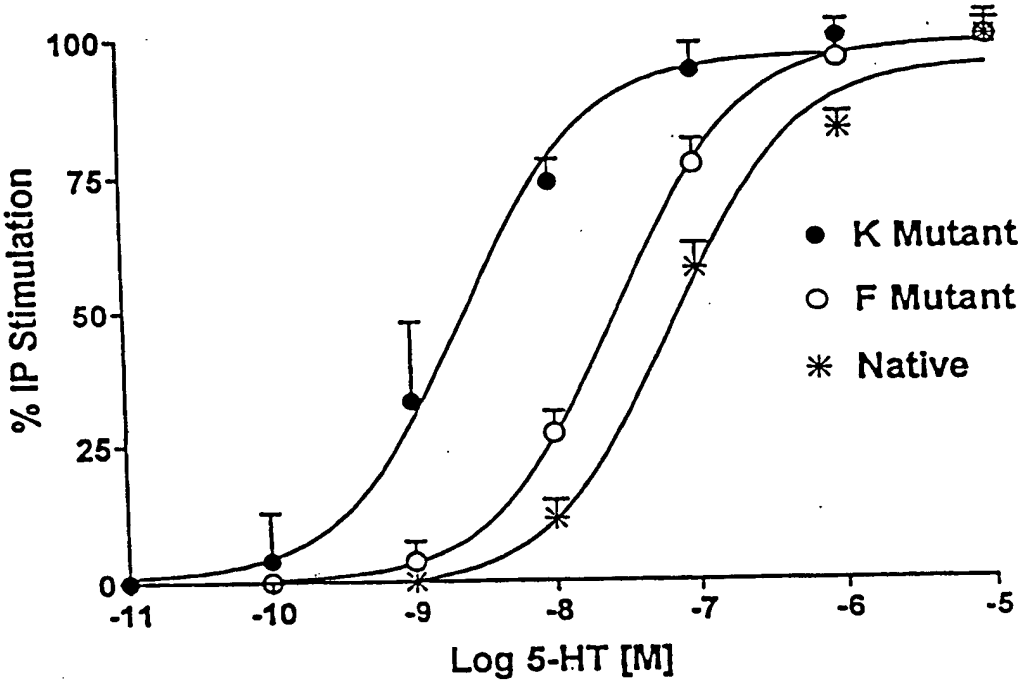

100
75
50
25
0
% IP Stimulation
-11
-10
-9
-8
-7
-6
-5
Log 5-HT [M]
K Mutant
F Mutant
Native

FIGURE 14

5-HT_{2C} Receptor	5-HT EC_{50} (nM)	K_D (nM)	Bmax (pm/mg)
Native	70+/-18	0.6+/-0.1	1.5+/-0.2
F Mutant	28+/-2.5*	1.3+/-0.2*	0.6+/-0.1*
K Mutant	2.7+/-1.1*	1.2+/-0.1*	1.4+/-0.2

FIGURE 15

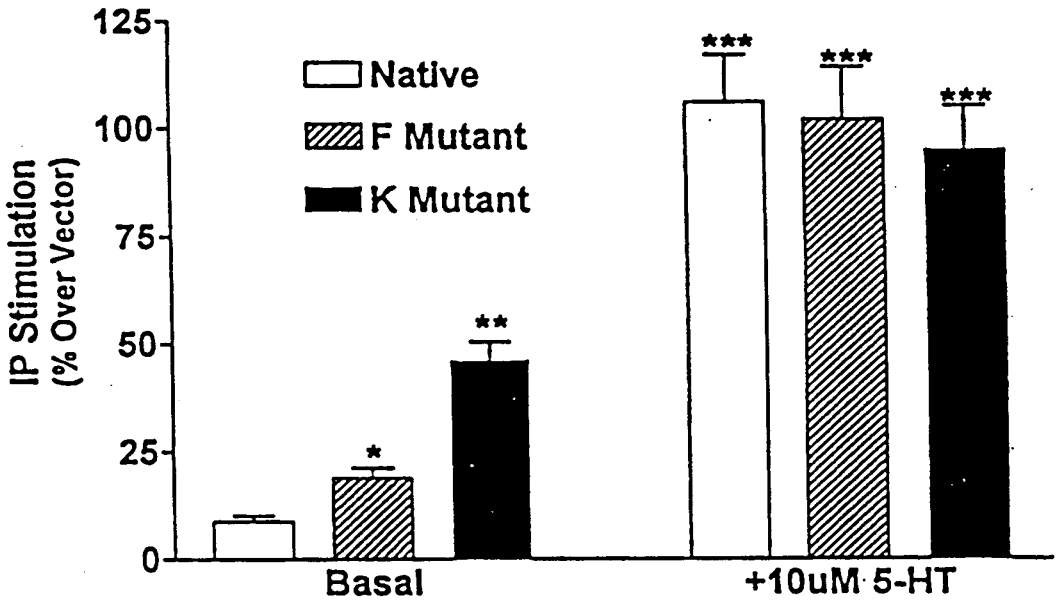

125
100
75
50
25
0
IP Stimulation
(% Over Vector)
Native
F Mutant
K Mutant
*
**
***
***
***
Basal
+10uM 5-HT

FIGURE 16

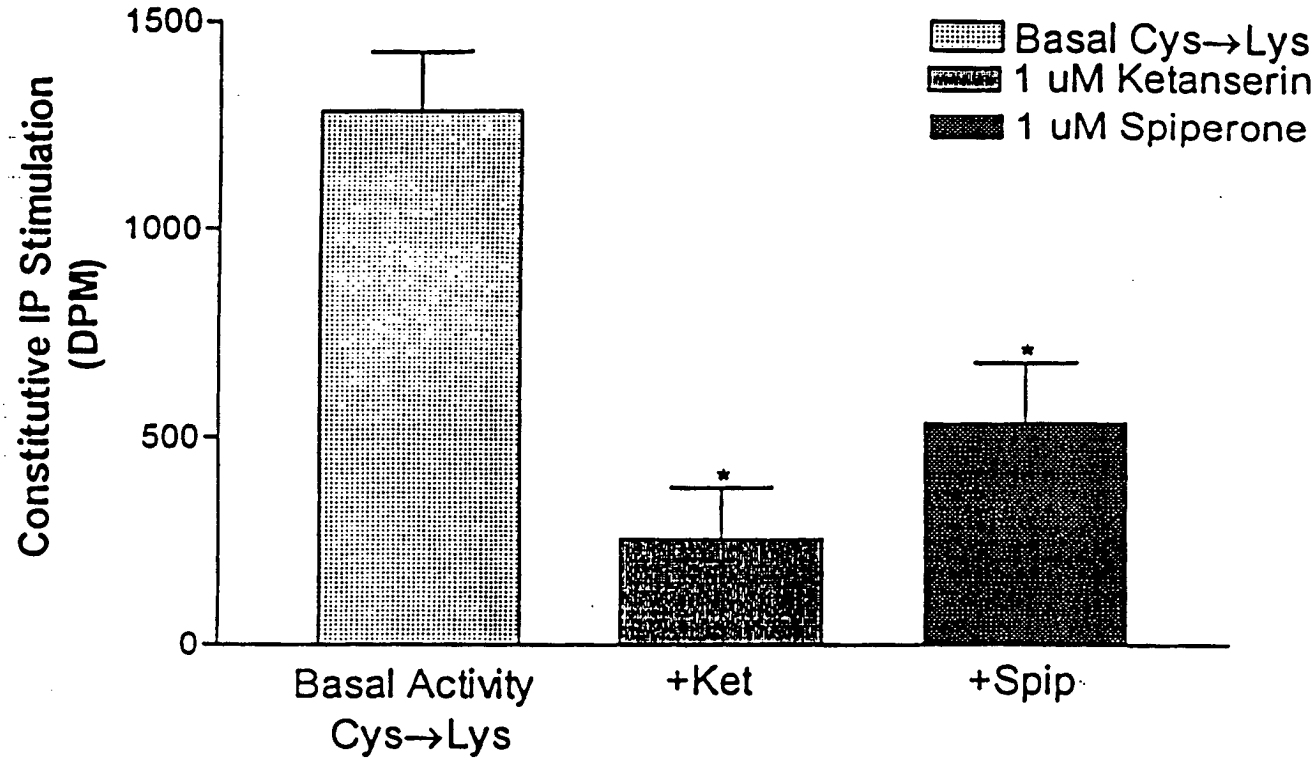

Constitutive IP Stimulation (DPM)
1500
1000
500
0
Basal Cys→Lys
1 uM Ketanserin
1 uM Spiperone
*
*
Basal Activity Cys→Lys
+Ket
+Spip

FIGURE 17

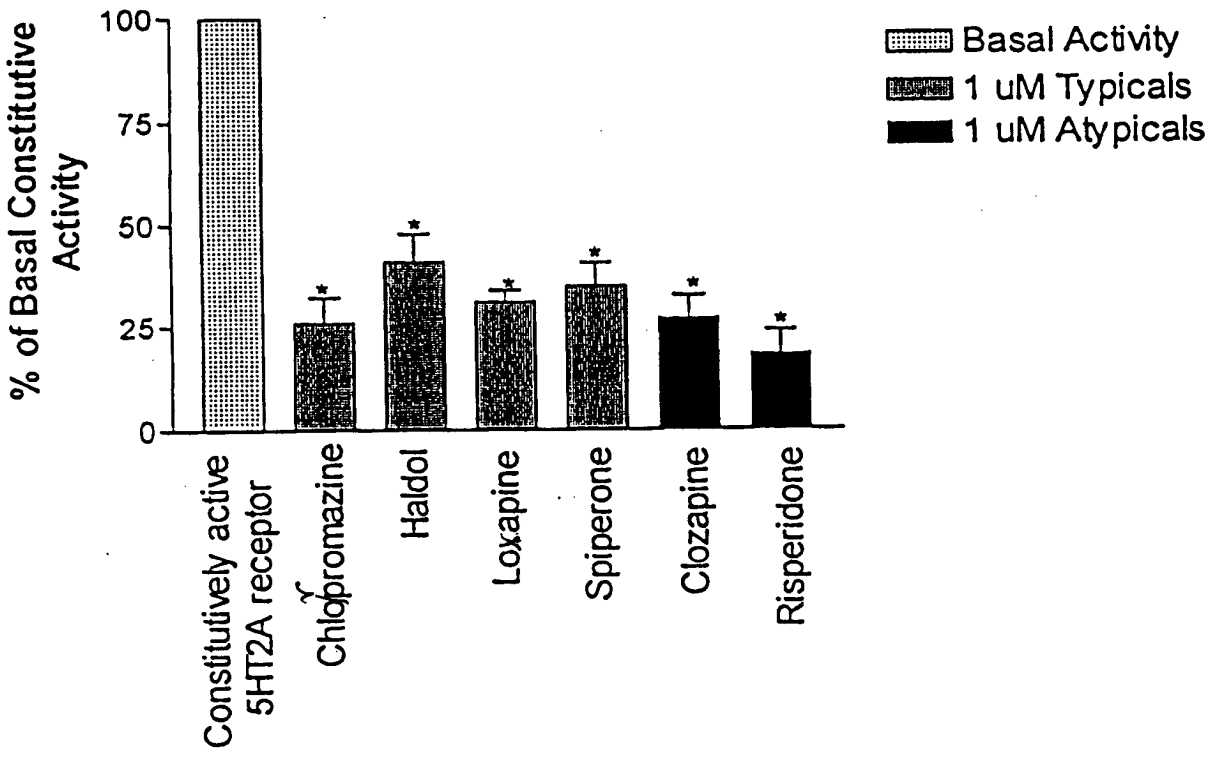

% of Basal Constitutive Activity
100
75
50
25
0
Basal Activity
1 uM Typicals
1 uM Atypicals
Constitutively active 5HT2A receptor
Chlorpromazine
Haldol
Loxapine
Spiperone
Clozapine
Risperidone

FIGURE 18

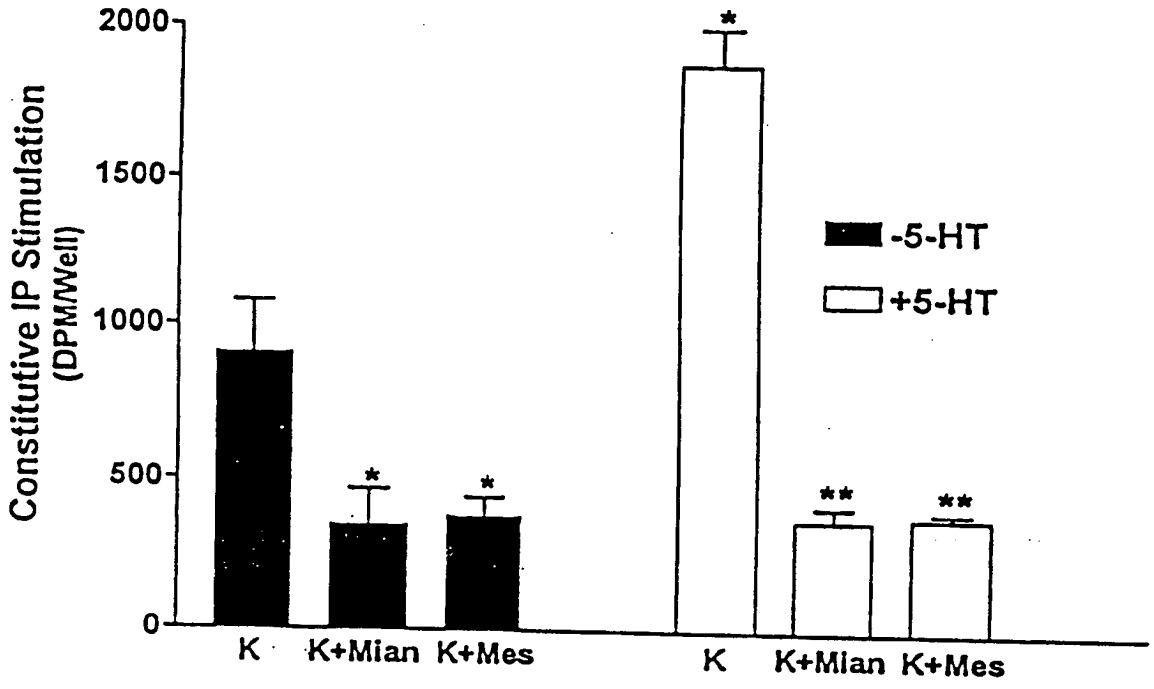

Constitutive IP Stimulation
(DPM/Well)
2000
1500
1000
500
0
*
*
*
**
**
-5-HT
+5-HT
K
K+Mian
K+Mes
K
K+Mian
K+Mes

FIGURE 19

Human 5-HT$_{2A}$

1 gaattcgggt gagccagctc cgggagaaca gcatgtacac cagcctcagt gttacagagt

61 gtgggtacat caaggtgaat ggtgagcaga aactataacc tgttagtcct tctacacctc

121 atctgctaca agttctggct tagacatgga tattctttgt gaagaaaata cttctttgag

181 ctcaactacg aactccctaa tgcaattaaa tgatgacacc aggctctaca gtaatgactt

241 taactctgga gaagctaaca cttctgatgc atttaactgg acagtcgact ctgaaaatcg

301 aaccaacctt tcctgtgaag ggtgcctctc accgtcgtgt ctctccttac ttcatctcca

361 ggaaaaaaac tggtctgctt tactgacagc cgtagtgatt attctaacta ttgctggaaa

421 catactcgtc atcatggcag tgtccctaga gaaaaagctg cagaatgcca ccaactattt

481 cctgatgtca cttgccatag ctgatatgct gctgggtttc cttgtcatgc ccgtgtccat

541 gttaaccatc ctgtatgggt accggtggcc tctgccgagc aagctttgtg cagtctggat

601 ttacctggac gtgctcttct ccacggcctc catcatgcac ctctgcgcca tctcgctgga

661 ccgctacgtc gccatccaga atcccatcca ccacagccgc ttcaactcca gaactaaggc

721 atttctgaaa atcattgctg tttggaccat atcagtaggt atatccatgc caataccagt

781 ctttgggcta caggacgatt cgaaggtctt taaggagggg agttgcttac tcgccgatga

841 taactttgtc ctgatcggct cttttgtgtc atttttcatt cccttaacca tcatggtgat

901 cacctacttt ctaactatca agtcactcca gaaagaagct actttgtgtg taagtgatct

961 tggcacacgg gccaaattag cttctttcag cttcctccct cagagttctt tgtcttcaga

1021 aaagctcttc cagcggtcga tccataggga gccagggtcc tacacaggca ggaggactat

1081 gcagtccatc agcaatgagc aaaaggcatg caaggtgctg ggcatcgtct tcttcctgtt

1141 tgtggtgatg tggtgccctt tcttcatcac aaacatcatg gccgtcatct gcaaagagtc

1201 ctgcaatgag gatgtcattg gggccctgct caatgtgttt gtttggatcg gttatctctc

1261 ttcagcagtc aacccactag tctacacact gttcaacaag acctataggt cagccttttc

FIGURE 20A

1321 acggtatatt cagtgtcagt acaaggaaaa caaaaaacca ttgcagttaa ttttagtgaa

1381 cacaataccg gctttggcct acaagtctag ccaacttcaa atgggacaaa aaaagaattc

1441 aaagcaagat gccaagacaa cagataatga ctgctcaatg gttgctctag gaaagcagca

1501 ttctgaagag gcttctaaag acaatagcga cggagtgaat gaaaaggtga gctgtgtgtg

1561 ataggctagt tgccgtggca actgtggaag gcacactgag caagttttca cctatctgga

1621 aaaaaaaaat atgagattgg aaaaaattag acaagtctag tggaaccaac gatcatatct

1681 gtatgcctca ttttattctg tcaatgaaaa gcggggttca atgctacaaa atgtgtgctt

1741 ggaaaatgtt ctgacagcat ttcagctgtg agctttctga tacttattta taacattgta

1801 aatgatatgt ctttaaaatg attcactttt attgtataat tatgaagccc taagtaaatc

1861 taaattaact tctattttca agtggaaacc ttgctgctat gctgttcatt gatgacatgg

1921 gattgagttg gttacctatt gccgtaaata aaaatagcta taaatagtga aaattttatt

1981 gaatataatg gcctcttaaa aattatcttt aaaacttact atggtatata ttttgaaagg

2041 agaaaaaaaa aaagccacta aggtcagtgt tataaaatct gtattgctaa gataattaaa

2101 tgaaatactt gacaacattt ttcatagata ccattttgaa atattcacaa ggttgctggc

2161 atttgctgca tttcaagtta attctcagaa gtgaaaaaga cttcaaatgt tattcaataa

2221 ctattgctgc tttctcttct acttcttgtg ctttactctg aatttccagt gtggtcttgt

2281 ttaatatttg ttcctctagg taaactagca aaaggatgat ttaacattac caaatgcctt

2341 tctagcaatt gcttctctaa aacagcacta tcgaggtatt tggtaacttg ctgtgaaatg

2401 actgcatcat gcatgcactc ttttgagcag taaatgtata ttgatgtaac tgtgtcagga

2461 ttgaggatga actcaggttt ccggctactg acagtggtag agtcctagga catctctgta

2521 aaaagcaggt gactttccta tgacactcat caggtaaact gatgctttca gatccatcgg

2581 tttatactat ttattaaaac cattctgctt ggttccacaa tcatctattg agtgtacatt

2641 tatgtgtgaa gcaaatttct agatatgaga aatataaaaa taattaaaac aaaatccttg

FIGURE 20A - CONTINUED

BNSDOCID: <WO 9838217A1 | >

2701 ccttcaaacg aaatggctcg gccaggcacg gaggctcgtg catgtaatcc tagcactttg

2761 ggaggctgag atgggaggat cacttgaggc caagagtttg agaccaacct gggtaacaaa

2821 gtgagacctc cctgtctcta caaaaaaaat caaaaaatta tctgatcctt gtggcacaca

2881 actgtggtcc cagctacagg ggaggctgag acgcaaggat cacttgagcc cagaagctca

2941 aggctgcagt gagccaagtt cacaccactg ccatttcctc ctgggcaaca gagtgagacc

3001 ctatcacccc gaattc

FIGURE 20A - CONTINUED

Human 5-HT2A

MDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFN

WTVDSENRTNLSCEGCLSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSISNEQKACKVLGIVFFLFVV

MWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDAKTTDNDCSMVALGK

QHSEEASKDNSDGVNEKVSCV

FIGURE 20B

Human 5-HT_{2c}

1 gaattcggga gcgtcctcag atgcaccgat cttcccgata ctgcctttgg agcggctaga

61 ttgctagcct tggctgctcc attggcctgc cttgcccctt acctgccgat tgcatatgaa

121 ctcttcttct gtctgtacat cgttgtcgtc ggagtcgtcg cgatcgtcgt ggcgctcgtg

181 tgatggcctt cgtccgttta gagtagtgta gttagttagg ggccaacgaa gaagaaagaa

241 gacgcgatta gtgcagagat gctggaggtg gtcagttact aagctagagt aagatagcgg

301 agcgaaaaga gccaaaccta gccgggggggc gcacggtcac ccaaaggagg tcgactcgcc

361 ggcgcttcct atcgcgccga gctccctcca ttcctctccc tccgccgagg cgcgaggttg

421 cggcgcgcag cgcagcgcag ctcagcgcac cgactgccgc gggctccgct gggcgattgc

481 agccgagtcc gtttctcgtc tagctgccgc cgcggcgacc gctgcctggt cttcctcccg

541 gacgctagtg ggttatcagc taacacccgc gagcatctat aacataggcc aactgacgcc

601 atccttcaaa aacaactgtc tgggaaaaaa agaataaaaa gtagtgtgag agcagaaaac

661 gtgattgaaa cacgaccaat ctttcttcag tgccaaaggg tggaaaagaa aggatgatat

721 gatgaaccta gcctgttaat ttcgtcttct caattttaaa ctttggttgc ttaagactga

781 agcaatcatg gtgaacctga ggaatgcggt gcattcattc cttgtgcacc taattggcct

841 attggtttgg caatgtgata tttctgtgag cccagtagca gctatagtaa ctgacatttt

901 caatacctcc gatggtggac gcttcaaatt cccagacggg gtacaaaact ggccagcact

961 ttcaatcgtc atcataataa tcatgacaat aggtggcaac atccttgtga tcatggcagt

1021 aagcatggaa aagaaactgc acaatgccac caattacttc ttaatgtccc tagccattgc

1081 tgatatgcta gtgggactac ttgtcatgcc cctgtctctc ctggcaatcc tttatgatta

1141 tgtctggcca ctacctagat atttgtgccc cgtctggatt tctttagatg ttttatttttc

1201 aacagcgtcc atcatgcacc tctgcgctat atcgctggat cggtatgtag caatacgtaa

1261 tcctattgag catagccgtt tcaattcgcg gactaaggcc atcatgaaga ttgctattgt

FIGURE 21A

1321 ttgggcaatt tctataggtg tatcagttcc tatccctgtg attggactga gggacgaaga

1381 aaaggtgttc gtgaacaaca cgacgtgcgt gctcaacgac ccaaatttcg ttcttattgg

1441 gtccttcgta gctttcttca taccgctgac gattatggtg attacgtatt gcctgaccat

1501 ctacgttctg cgccgacaag ctttgatgtt actgcacggc cacaccgagg aaccgcctgg

1561 actaagtctg gatttcctga agtgctgcaa gaggaatacg gccgaggaag agaactctgc

1621 aaaccctaac caagaccaga acgcacgccg aagaaagaag aaggagagac gtcctagggg

1681 caccatgcag gctatcaaca atgaaagaaa agcttcgaaa gtccttggga ttgttttctt

1741 tgtgtttctg atcatgtggt gcccattttt cattaccaat attctgtctg ttctttgtga

1801 gaagtcctgt aaccaaaagc tcatggaaaa gcttctgaat gtgtttgttt ggattggcta

1861 tgtttgttca ggaatcaatc ctctggtgta tactctgttc aacaaaattt accgaagggc

1921 attctccaac tatttgcgtt gcaattataa ggtagagaaa aagcctcctg tcaggcagat

1981 tccaagagtt gccgccactg ctttgtctgg gagggagctt aatgttaaca tttatcggca

2041 taccaatgaa ccggtgatcg agaaagccag tgacaatgag cccggtatag agatgcaagt

2101 tgagaattta gagttaccag taaatccctc cagtgtggtt agcgaaagga ttagcagtgt

2161 gtgagaaaga acagcacagt cttttctacg gtacaagcta catatgtagg aaaattttct

2221 tctttaattt ttctgttggt cttaactaat gtaaatattg ctgtctgaaa aagtgttttt

2281 acatatagct ttgcaacctt gtactttaca atcatgccta cattagtgag atttagggtt

2341 ctatatttac tgtttataat aggtggagac taacttattt tgattgtttg atgaataaaa

2401 tgtttatttt tgctctccct cccttctttc cttccttttt tcctttcttc cttcctttct

2461 ctctttcttt tgtgcatatg gcaacgttca tgttcatctc aggtggcatt tgcaggtgac

2521 cagaatgagg cacatgacag tggttatatt tcaaccacac ctaaattaac aaattcagtg

2581 gacatttgtt ctgggttaac agtaaatata cactttacat tcttgctctg ctcatctaca

2641 catataaaca cagtaagata ggttctgctt tctgatacat ctgtcagtga gtcagaggca

FIGURE 21A - CONTINUED

2701 gaacctagtc ttgttgttca tataggggaa ttc

FIGURE 21A - CONTINUED

Human 5-HT_{2C}

MVNLRNAVHSFLVHLIGLLVWQCDISVSPVAAIVTDIFNTSDGG

RFKFPDGVQNWPALSIVIIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLV

GLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPI

EHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDEEKVFVNNTTCVLNDPNFVLIG

SFVAFFIPLTIMVITYCLTIYVLRRQALMLLHGHTEEPPGLSLDFLKCCKRNTAEEEN

SANPNQDQNARRRKKKERRPRGTMQAINNERKASKVLGIVFFVFLIMWCPFFITNILS

VLCEKSCNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSNYLRCNYKVEKK

PPVRQIPRVAATALSGRELNVNIYRHTNEPVIEKASDNEPGIEMQVENLELPVNPSSV

VSERISSV

FIGURE 21B

Rat 5-HT_{2A} Cysteine → Lysine Mutant

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKAKKVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 22

Rat $5HT_{2A}$ Cysteine → Lysine Mutant

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322K primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322K primer

1021 cgaaggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 23

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 23 - CONTINUED

Rat $5HT_{2A}$ Cysteine → Lysine Mutant with Restriction Site

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

Start C322K primer

End C322K primer

1021 cgaaagaaagt actgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

Mutations to create Sca1 site

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 24

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agaggaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 24 - CONTINUED

Rat 5-HT_{2A} Cysteine → Arginine Mutant

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKA[R]KVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 25

Rat $5HT_{2A}$ Cysteine → Arginine Mutant

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcatttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322R primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322R primer

1021 cgaggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 26

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 26 - CONTINUED

Rat $5HT_{2A}$ Cysteine → Arginine Mutant with Restriction Site

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322R primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322R primer

1021 cgaggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 27

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 27 - CONTINUED

Rat 5-HT_{2A} Cysteine → Glutamic Acid Mutant

MEILCEDNISLSSIPNSLMQLGDGPRLYHNDFNSRDANTSEASN

WTIDAENRTNLSCEGYLPPTCLSILHLQEKNWSALLTTVVIILTIAGNILVIMAVSLE

KKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAIWIYLDVLFST

ASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDD

SKVFKEGSCLLADDNFVLIGSFVAFFIPLTIMVITYFLTIKSLQKEATLCVSDLSTRA

KLASFSFLPQSSLSSEKLFQRSIHREPGSYAGRRTMQSISNEQKAEKVLGIVFFLFVV

MWCPFFITNIMAVICKESCNENVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFS

RYIQCQYKENRKPLQLILVNTIPALAYKSSQLQVGQKKNSQEDAEQTVDDCSMVTLGK

QQSEENCTDNIETVNEKVSCV

FIGURE 28

Rat $5HT_{2A}$ Cysteine → Glutamic Acid Mutant

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagaagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322E primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322E primer

1021 cggaggaaggt gctgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 29

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 29 - CONTINUED

Rat $5HT_{2A}$ Cysteine → Glutamic Acid Mutant with Restriction Site

1 cccaggctat gaacccctag tctctccaca cttcatctgc tacaacttcc ggcttagaca

61 tggaaattct ttgtgaagac aatatctctc tgagctcaat tccaaactcc ttaatgcaat

121 taggtgatgg cccgaggctc taccataatg acttcaactc cagagatgct aacacttcgg

181 aagcatcgaa ctggacaatt gatgctgaaa acagaaccaa cctctcctgt gaagggtacc

241 tcccaccgac atgcctctcc attcttcatc tccaggaaaa aaactggtct gctttattga

301 caactgtcgt gattattctc accattgctg gaaatatact ggtcatcatg gcagtgtccc

361 tagaaaaaaa gctgcagaat gccaccaact atttcctgat gtcacttgcc atagctgata

421 tgctgctggg tttccttgtc atgcctgtgt ccatgttaac catcctgtat gggtaccggt

481 ggcctttgcc tagcaagctc tgtgcgatct ggatttacct ggatgtgctc ttttctacgg

541 catccatcat gcacctctgc gccatctccc tggaccgcta tgtcgccatc cagaacccca

601 ttcaccacag ccgcttcaac tccagaacca aagccttcct gaaaatcatt gccgtgtgga

661 ccatatctgt aggtatatcc atgccaatcc cagtctttgg actacaggat gattcgaagg

721 tctttaagga ggggagctgc ctgcttgccg atgacaactt tgttctcata ggctcttttg

781 tggcattttt catcccccta accatcatgg tgatcaccta cttcctgact atcaagtcac

841 ttcagaaaga agccaccttg tgtgtgagtg acctcagcac tcgagccaaa ctagcctcct

901 tcagcttcct ccctcagagt tctctgtcat cagaaaagct cttccaacgg tccatccaca

Start C322E primer

961 gagagccagg ctcctacgca ggccgaagga cgatgcagtc catcagcaat gagcaaaagg

End C322E primer

1021 cggagaaggt actgggcatc gtgttcttcc tgtttgttgt aatgtggtgc ccattcttca

Mutation to create Rsa1 site

1081 tcaccaatat catggccgtc atctgcaaag aatcctgcaa tgaaaatgtc atcggagccc

1141 tgctcaatgt gtttgtctgg attggttatc tctcctcagc tgtcaatcca ctggtatata

1201 cgttgttcaa taaaacttat aggtccgcct tctcaaggta cattcagtgt cagtacaagg

1261 aaaacagaaa gccactgcag ttaattttag tgaacactat accagcattg gcctacaagt

FIGURE 30

1321 ctagtcagct ccaggtggga cagaaaaaga actcacagga agatgctgag cagacagttg

1381 atgactgctc catggttaca ctggggaaac aacagtcgga agagaattgt acagacaata

1441 ttgaaaccgt gaatgaaaag gttagctgtg tgtgatgaac tggatgctat ggcaattgcc

1501 cagggcatgt gaacaaggtt atacccatgt gtgtggggcg gggataagga ggctgcaaca

1561 aattag

FIGURE 30 - CONTINUED

Rat 5-HT_{2c} Serine → Lysine Mutant

MVNLGNAVRSLLMHLIGLLVWQFDISISPVAAIVTDTFNSSDGG

RLFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADML

VGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNP

IEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLI

GSFVAFFIPLTIMVITYFLTIYVLRRQTLMLLRGHTEEELANMSLNFLNCCCKKNGGE

EENAPNPNPDQKPRRKKKEKRPRGTMQAINNEKKAKKVLGIVFFVFLIMWCPFFITNI

LSVLCGKACNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSKYLRCDYKPD

KKPPVRQIPRVAATALSGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS

NVVSERISSV

FIGURE 31

Rat $5HT_{2c}$ Serine → Lysine Mutant

ORIGIN 23 bp upstream of HindIII site.

1 ggcgctctgg tgctcactga ggaagcttcc ttaggtgtac cgatcttaat gattgagccc

61 ttggagcagc aagattgtta atcttggttg ctcctttggc ctgtctatcc cttaccttcc

121 tattacatat gaacttttct tcgttctgca catcgattgt cgtcggcgtc gtggagatcg

181 tcgtggtgct ccggtggtgg tcttcgtccg cttagaatag tgtagttagt taggggcctt

241 caaagaagaa agaagaagcg attggcgcgg agagatgctg gaggtgtcag tttctatgct

301 agagtagggt agtgaaacaa tccccagcca aacctttccg gggggcgcag gttgcccaca

361 ggaggtcgac ttgccggcgc tgtccttcgc gccgagctcc ctccatcctt ctttccgtct

421 gctgagacgc aaggttgcgg cgcgcacgct gagcagcgca ctgactgccg cgggctccgc

481 tgggcgattg cagccgagtc cgtttctcgt ctagctgccg ccgcggcgac ctgcctggtc

541 ttcctcccgg acgctagcgg gttgtcaact attacctgca agcataggcc aacgaacacc

601 ttctttccaa attaattgga atgaaacaat tctgttaact tcctaattct cagtttgaaa

661 ctctggttgc ttaagcctga agcaatcatg gtgaaccttg gcaacgcggt gcgctcgctc

721 ctgatgcacc taatcggcct attggtttgg caattcgata tttccataag tccagtagca

781 gctatagtaa ctgacacttt taattcctcc gatggtggac gcttgtttca attcccggac

841 ggggtacaaa actggccagc actttcaatc gtcgtgatta taatcatgac aataggggcc

901 aacattcttg ttatcatggc agtaagcatg gagaagaaac tgcacaatgc aaccaattac

961 ttcttaatgt ccctagccat tgctgatatg ctggtgggac tacttgtcat gcccctgtcc

1021 ctgcttgcta ttctttatga ttatgtctgg cctttaccta gatatttgtg ccccgtctgg

1081 atttcactag atgtgctatt ttcaactgcg tccatcatgc acctctgcgc catatcgctg

1141 gaccggtatg tagcaatacg taatcctatt gagcatagcc ggttcaattc gcggactaag

1201 gccatcatga agattgccat cgtttgggca atatcaatag gagtttcagt tcctatccct

FIGURE 32

1261 gtgattggac tgagggacga aagcaaagtg ttcgtgaata acaccacgtg cgtgctcaat

1321 gaccccaact tcgttctcat cgggtccttc gtggcattct tcatcccgtt gacgattatg

1381 gtgatcacct acttcttaac gatctacgtc ctgcgccgtc aaactctgat gttacttcga

1441 ggtcacaccg aggaggaact ggctaatatg agcctgaact ttctgaactg ctgctgcaag

1501 aagaatggtg gtgaggaaga gaacgctccg aaccctaatc cagatcagaa accacgtcga

Start S312K primer

1561 aagaagaaag aaaagcgtcc cagaggcacc atgcaagcta tcaacaacga aaagaaagct

End S312K primer

1621 aagaaagtcc ttggcattgt attctttgtg tttctgatca tgtggtgccc gtttttcatc

1681 accaatatcc tgtcggttct ttgtgggaag gcctgtaacc aaaagctaat ggagaagctt

1741 ctcaatgtgt ttgtgtggat tggctatgtg tgttcaggca tcaatcctct ggtgtacact

1801 ctctttaata aaatttaccg aagggctttc tctaaatatt tgcgctgcga ttataagcca

1861 gacaaaaagc ctcctgttcg acagattcct agggttgctg ccactgcttt gtctgggagg

1921 gagctcaatg ttaacattta tcggcatacc aatgaacgtg tggctaggaa agctaatgac

1981 cctgagcctg gcatagagat gcaggtggag aacttagagc tgccagtcaa cccctctaat

2041 gtggtcagcg agaggattag tagtgtgtaa gcgaagagca gcgcagactt cctacaggaa

2101 agttcctgta ggaaagtcct ccccaccccc cgtgattttc ctgtgaatca taactaatgt

2161 aaatattgct gtgtgacaag acagtgtttt tataaatagc tttgcaaccc tgtactttac

2221 atcatgcgtt aatagtgaga ttcggg

FIGURE 32 - CONTINUED

Rat $5HT_{2c}$ Serine → Lysine Mutant with Restriction Site

ORIGIN 23 bp upstream of HindIII site.

1 ggcgctctgg tgctcactga ggaagcttcc ttaggtgtac cgatcttaat gattgagccc

61 ttggagcagc aagattgtta atcttggttg ctcctttggc ctgtctatcc cttaccttcc

121 tattacatat gaacttttct tcgttctgca catcgattgt cgtcggcgtc gtggagatcg

181 tcgtggtgct ccggtggtgg tcttcgtccg cttagaatag tgtagttagt taggggcctt

241 caaagaagaa agaagaagcg attggcgcgg agagatgctg gaggtgtcag tttctatgct

301 agagtagggt agtgaaacaa tccccagcca aacctttccg gggggcgcag gttgcccaca

361 ggaggtcgac ttgccggcgc tgtccttcgc gccgagctcc ctccatcctt ctttccgtct

421 gctgagacgc aaggttgcgg cgcgcacgct gagcagcgca ctgactgccg cgggctccgc

481 tgggcgattg cagccgagtc cgtttctcgt ctagctgccg ccgcggcgac ctgcctggtc

541 ttcctcccgg acgctagcgg gttgtcaact attacctgca agcataggcc aacgaacacc

601 ttctttccaa attaattgga atgaaacaat tctgttaact tcctaattct cagtttgaaa

661 ctctggttgc ttaagcctga agcaatcatg gtgaaccttg gcaacgcggt gcgctcgctc

721 ctgatgcacc taatcggcct attggtttgg caattcgata tttccataag tccagtagca

781 gctatagtaa ctgacacttt taattcctcc gatggtggac gcttgtttca attcccggac

841 ggggtacaaa actggccagc actttcaatc gtcgtgatta taatcatgac aatagggggc

901 aacattcttg ttatcatggc agtaagcatg gagaagaaac tgcacaatgc aaccaattac

961 ttcttaatgt ccctagccat tgctgatatg ctggtgggac tacttgtcat gcccctgtcc

1021 ctgcttgcta ttctttatga ttatgtctgg cctttaccta gatatttgtg ccccgtctgg

1081 atttcactag atgtgctatt ttcaactgcg tccatcatgc acctctgcgc catatcgctg

1141 gaccggtatg tagcaatacg taatcctatt gagcatagcc ggttcaattc gcggactaag

1201 gccatcatga agattgccat cgtttgggca atatcaatag gagtttcagt tcctatccct

FIGURE 33

1261 gtgattggac tgagggacga aagcaaagtg ttcgtgaata acaccacgtg cgtgctcaat

1321 gaccccaact tcgttctcat cgggtccttc gtggcattct tcatcccgtt gacgattatg

1381 gtgatcacct acttcttaac gatctacgtc ctgcgccgtc aaactctgat gttacttcga

1441 ggtcacaccg aggaggaact ggctaatatg agcctgaact ttctgaactg ctgctgcaag

1501 aagaatggtg gtgaggaaga gaacgctccg aaccctaatc cagatcagaa accacgtcga

Start S312K primer

1561 aagaagaaag aaaagcgtcc cagaggcacc atgcaagcta tcaacaacga aaagaaagct

End S312K primer

1621 aaGaaaagtac ttggcattgt attctttgtg tttctgatca tgtggtgccc gtttttcatc

Mutation to create Sca1 site

1681 accaatatcc tgtcggttct ttgtgggaag gcctgtaacc aaaagctaat ggagaagctt

1741 ctcaatgtgt ttgtgtggat tggctatgtg tgttcaggca tcaatcctct ggtgtacact

1801 ctctttaata aaatttaccg aagggctttc tctaaatatt tgcgctgcga ttataagcca

1861 gacaaaaagc ctcctgttcg acagattcct agggttgctg ccactgcttt gtctgggagg

1921 gagctcaatg ttaacattta tcggcatacc aatgaacgtg tggctaggaa agctaatgac

1981 cctgagcctg gcatagagat gcaggtggag aacttagagc tgccagtcaa cccctctaat

2041 gtggtcagcg agaggattag tagtgtgtaa gcgaagagca gcgcagactt cctacaggaa

2101 agttcctgta ggaaagtcct ccccaccccc cgtgattttc ctgtgaatca taactaatgt

2161 aaatattgct gtgtgacaag acagtgtttt tataaatagc tttgcaaccc tgtactttac

2221 atcatgcgtt aatagtgaga ttcggg

FIGURE 33 - CONTINUED

BNSDOCID: <WO 9838217A1 >

Rat 5-HT_{2c} Serine → Phenylalanine Mutant

MVNLGNAVRSLLMHLIGLLVWQFDISISPVAAIVTDTFNSSDGG

RLFQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADML

VGLLVMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNP

IEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLI

GSFVAFFIPLTIMVITYFLTIYVLRRQTLMLLRGHTEEELANMSLNFLNCCCKKNGGE

EENAPNPNPDQKPRRKKKEKRPRGTMQAINNEKKAFKVLGIVFFVFLIMWCPFFITNI

LSVLCGKACNQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSKYLRCDYKPD

KKPPVRQIPRVAATALSGRELNVNIYRHTNERVARKANDPEPGIEMQVENLELPVNPS

NVVSERISSV

FIGURE 34

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(54) Title: MODIFIED G-PROTEIN COUPLED RECEPTORS

(57) Abstract

Modified G-protein coupled receptors having deletions in the third intracellular domain are identified and methods of making the modified receptors are provided. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and compounds identified through the use of the modified receptors, including modulators of the receptors.

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TITLE OF THE INVENTION
MODIFIED G-PROTEIN COUPLED RECEPTORS

CROSS RELATED TO OTHER APPLICATIONS

This is a continuation-in-part of U.S. Serial Number 08/267,987, filed June 29, 1994, now pending, and an continuation-in-part of U. S. Serial Number 08/267,987, filed June 29, 1994, now pending.

BACKGROUND OF THE INVENTION

G-protein coupled receptors are cell surface receptors that mediate the responses of the cell to a variety of environmental signals. Upon binding an agonist, the receptor interacts with one or more specific G proteins, which then regulate the activities of specific effector proteins. By this means, activation of G-protein coupled receptors amplifies the effects of the environmental signal and initiates a cascade of intracellular events that ultimately leads to a defined cellular response. The family of G-protein coupled receptors function as a complex information processing network within the plasma membrane of the cell, acting to coordinate a cell's response to multiple environmental signals.

G-protein coupled receptors are characterized by the ability of agonists to promote the formation of a high affinity ternary complex between the agonist, the receptor and the G-protein (Figure 1). The α-subunit of the G protein contains a guanine nucleotide binding site which, in the high affinity ternary [G protein-receptor-agonist] complex, is occupied by GDP. In the presence of physiological concentrations of GTP, the GDP molecule in the guanine nucleotide binding site of the G protein is displaced by a GTP molecule. The binding of GTP dissociates the α subunit of the G protein from its βγ subunits and from the receptor, thereby activating the G-protein to stimulate downstream effectors (adenylyl cyclase in the case of the β-adrenergic receptor (βAR)) and propagating the intracellular signal. Thus, the ternary complex is transient in the presence of physiological

GTP concentrations. Because the affinity of the agonist for the receptor-G protein complex is higher than its affinity for the uncomplexed receptor, one consequence of the destabilization of the ternary complex is a reduction in the affinity of the receptor for the agonist. Thus, the affinity of agonists for G-protein coupled receptors is a function of the efficiency with which the receptor is coupled to the G-protein. In contrast, antagonists bind with the same affinity to the receptor in the presence or absence of G-protein coupling.

The observation that agonist affinity can be reduced by conditions under which a receptor is not optimally coupled to its G-protein has important implications for the identification of agonists of G-protein coupled receptors, particularly identification based on ligand binding. If the receptor is not optimally coupled to the G-protein under the conditions of binding assays, an agonist will bind to the receptor with relatively low affinity. Thus, a screen that relies on a binding assay based on displacement of a radiolabeled ligand, although attractive for its ease and the potential for high throughput, poses the risk that a promising partial agonist might be overlooked because the agonist would bind predominantly to the low affinity state of the receptor, and thus would have low affinity in the binding assay. Consequently, functional assays are frequently used to screen for agonists of G-protein coupled receptors. However, functional assays (ranging from ex vivo muscle contraction assays to determination of second messenger levels in cells expressing exogenous cloned G-protein coupled receptors) are tedious and much more time-consuming than ligand binding assays, and hence are not readily adapted to high throughput screens. Because the modified receptors of the present invention bind agonists with high affinity in the presence or absence of the G-protein, they can be used in high throughput radioligand binding assays to screen for high affinity ligands, regardless of whether the ligands are agonists or antagonists.

G-protein coupled receptors consist of seven hydrophobic domains connecting eight hydrophilic domains. The hydrophobicity or hydrophilicity of the domains may be determined by standard hydropathy profiles, such as Kyte-Doolittle analysis (Kyte, J. and

Doolittle, R.J.F. *J. Mol. Biol.* 157: 105 (1982)). The receptors are thought to be oriented in the plasma membrane of the cell in such a way that the N-terminus of the receptor faces the extracellular space and the C-terminus of the receptor faces the cytoplasm, such that each of the hydrophobic domains crosses the plasma membrane. The receptors have been modeled and the putative boundaries of the extracellular, transmembrane and intracellular domains are generally agreed upon based on these models (for a review, see Baldwin, EMBO J. 12:1693, 1993). In general, the transmembrane domains are comprised of stretches of 20-25 amino acids in which most of the amino acid residues have hydrophobic side chains (including cysteine, methionine, phenylalanine, tyrosine, tryptophan, proline, glycine, alanine, valine, leucine, isoleucine), whereas the intracellular and extracellular loops are defined by contiguous stretches of several amino acids that have hydrophilic or polar side chains (including aspartate, glutamate, asparagine, glutamine, serine, threonine, histidine, lysine, and arginine). Polar amino acids, especially uncharged ones (such as serine, threonine, asparagine, and glutamine) are found in both transmembrane and extramembrane regions.

The extramembrane regions are characterized by contiguous stretches of three or more hydrophilic residues. In contrast, hydrophilic residues are found only in groups of 1-2, surrounded by hydrophobic residues, in the transmembrane domain. Thus, the transmembrane and extramembrane regions can be identified by the number of contiguous hydrophilic or hydrophobic amino acids in the primary sequence of the receptor, in addition to the constraints on the length of the hydrophobic segments given above. The boundaries between the transmembrane and extramembrane regions are often defined by the presence of charged or polar residues at the beginning or end of a stretch of hydrophobic amino acids. The locations of the mutations in the receptors of the present invention are described on the basis of these models and can be specifically defined by the specific amino acid numbers of the residues being mutated.

By these criteria, the third intracellular loop is defined as the hydrophilic loop connecting the hydrophobic, putative transmembrane domains V and VI. For example, in hamster β_2 adrenergic receptor, used to particularly exemplify the invention, the third intracellular loop would refer to amino acids 221 through 273 (Figure 2). In accordance with the principles described above, the beginning of this loop is defined by the presence of Arg_{221} (a charged residue at the end of the hydrophobic stretch of residues 198-220) and Lys_{273} (a charged residue at the beginning of the hydrophobic stretch of residues 274-298).

The present invention pertains to modified G-protein coupled receptors having deletions in the third intracellular domain. Methods of designing and making modified receptors are provided. The modified receptors are uncoupled from or are poorly coupled to their respective G-proteins. However, these modified receptors bind agonists with high affinity in the absence of G protein coupling. Because of their high intrinsic affinity for agonists, these modified receptors may be used in high throughput binding assays to identify compounds that bind to the receptor with high affinity, regardless of whether these compounds are agonists or antagonists. The invention includes the DNA encoding the modified receptors, the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and substances identified through the use of the modified receptors including specific modulators of the modified receptors. Modulators identified in this process are useful as therapeutic agents. Modulators, as described herein, include but are not limited to agonists, antagonists, suppressors and inducers.

SUMMARY OF THE INVENTION

Modified G-protein coupled receptors having deletions in the third intracellular domain are identified and methods of making the modified receptors are provided. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and compounds identified

through the use of the modified receptors, including modulators of the receptors. Modulators identified in this process are useful as therapeutic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic diagram of G-protein signal transduction system. The receptor is shown as a seven-helical bundle. α, ß, and γ indicate the three subunits of the G protein. E indicates an effector enzyme, such as adenylyl cyclase. The agonist (A) binding with high affinity to the receptor-G protein complex and with low affinity to the receptor alone is shown.

Figure 2. Schematic diagram of the hamster β2 adrenergic receptor. The third intracellular loop comprises residues 221-273. The proximal and distal segments of this loop are drawn in cylinders.

Figure 3. Stimulation of cAMP production as a function of isoproterenol by the wild type β3AR (closed circles) but not the modified D(227-234) (triangles) or D(277-289)β3AR (squares).

Figure 4: Binding of an agonist and an antagonist to the wild type (open circles) and D(277-289) β3AR (closed circles). Binding of the agonist isoproterenol (top panel) or the antagonist propranolol (bottom panel) was measured in competition with the radioligand ^{125}I-cyanopindolol.

Figure 5. Inhibition of adenylyl cyclase activity. A concentration dependent response curve of the ability of 5-HT to inhibit adenylate cyclase activity mediated by the wild type 5-HT1Dβ receptor is shown. However, in the histogram on the right of the figure, the inability of 100 mM 5-HT activating at the mutant receptor, D(231-239)5-HT1Dβ to produce an inhibition of adenylate cyclase activity is demonstrated. The results shown are from a typical experiment and were repeated three times and are representative of three independent mutant receptor cell lines [D(231-239)5-HT1Dβ clones 1, 21 and 65]. Formation of

^{32}P-cAMP from ^{32}P-ATP was measured in crude membrane preparations prepared from CHO cells stably expressing the appropriate receptors.

Figure 6. Table 1: Binding and functional parameters of the wild type and modified β_2AR.

Figure 7. Table 2: Binding parameters of the wild type and modified β_3AR.

Figure 8. Table 3: Radioligand binding properties of modified 5HT-1Dβ receptors. Presented in the table are the specific binding values (dpm) of 2 nm [^{3}H]5-HT observed in the presence and absence of the guanine nucleotide analog, GppNHp (100 mM). Also shown is the percentage inhibition of adenylate cyclase activity (%AC inhibition) for the respective cell lines. Results shown are from a typical experiment and were repeated three times.

DETAILED DESCRIPTION OF THE INVENTION

Modified G-protein coupled receptors having deletions in the third intracellular domain are identified and methods of making the modified receptors are provided. The modified receptors are uncoupled from or are poorly coupled to their respective G-proteins and may be used in assays to identify substances that bind to the receptor regardless of whether these substances are agonists or antagonists. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and compounds identified through the use of the modified receptors, including modulators of the receptors. Modulators identified in this process are useful as therapeutic agents. Modulators, as described herein, include but are not limited to agonists, antagonists, suppressors and inducers.

The term "G-protein coupled receptor" refers to any receptor protein that mediates its endogenous signal transduction

through activation of one or more guanine nucleotide binding regulatory proteins (G-proteins). These receptors share common structural features, including seven hydrophobic transmembrane domains. G-protein coupled receptors include receptors that bind to small biogenic amines, including but not limited to beta-adrenergic receptors (βAR), alpha-adrenergic receptors (αAR) and muscarinic receptors, as well as receptors whose endogenous ligands are peptides, such as neurokinin and glucagon receptors. Examples of βAR include beta-1, beta-2, and beta-3 adrenergic receptors. Examples of αAR include alpha-1a, alpha-1b, alpha-1c, alpha-2a, alpha-2b, and alpha-2c. Examples of muscarinic receptors include M1, M2, M3, M4 and M5. Examples of neurokinin receptors include NK1, NK2 and NK3. Other examples of G-protein coupled receptors include but are not limited to adenosine 2 receptor, alpha-2 adrenergic receptors, type-1 angiotensin II receptor, cholecytokinin B receptor, gastrin receptor, somatostatin receptor, 5-hydroxytryptamine 1 beta receptor, A2 adenosine receptor, Burkitt's lymphoma receptor, neuropeptide Y receptor, tachykinin receptor, serotonin receptor, formyl peptide receptor like-1, tyramine receptor, muscarinic acetylcholine receptor, certain endothelin receptors, complement protein 5a receptor, choriogonadotropic hormone receptor, high affinity interleukin 8 receptor, follicle stimulating hormone receptor, dopamine D1 receptor, C5a anaphylotoxin receptor, histamine H2 receptor, substance P receptor, thyrotropin receptor and, luteininzing hormone receptor. G-protein coupled receptors have been isolated from a variety of animals, including but not limited to humans, cows, goats, mice, pigs and rats.

Modified eceptors may include genetic variants, both natural and induced. Induced modified receptors may be derived by a variety of methods, including but not limited to, site-directed mutagenesis. Techniques for nucleic acid and protein manipulation are well-known in the art and are described generally in Methods in Enzymology and in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1989).

It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally-occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of a modified receptor is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of the modified receptor. The term "functional derivative" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of modified receptors. The term "fragment" is meant to refer to any polypeptide subset of modified receptors. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire modified receptor molecule or to a fragment thereof. A molecule is "substantially similar" to a modified receptor if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity,

they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical.

The term "analog" refers to a molecule substantially similar in function to either the entire modified receptor molecule or to a fragment thereof.

"Substantial homology" or "substantial similarity", when referring to nucleic acids means that the segments or their complementary strands, when optimally aligned and compared, are identical with appropriate nucleotide insertions or deletions, in at least 75% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize to a strand or its complement.

The nucleic acids claimed herein may be present in whole cells or in cell lysates or in a partially purified or substantially purified form. A nucleic acid is considered substantially purified when it is purified away from environmental contaminants. Thus, a nucleic acid sequence isolated from cells is considered to be substantially purified when purified from cellular components by standard methods while a chemically synthesized nucleic acid sequence is considered to be substantially purified when purified from its chemical precursors.

Nucleic acid compositions of this invention may be derived from genomic DNA or cDNA, prepared by synthesis or by a combination of techniques.

The natural or synthetic nucleic acids encoding the modified G-coupled protein receptors of the present invention may be incorporated into expression vectors. Usually the expression vectors incorporating the modified receptors will be suitable for replication in a host. Examples of acceptable hosts include, but are not limited to, prokaryotic and eukaryotic cells.

The phrase "recombinant expression system" as used herein means a substantially homogenous culture of suitable host organisms that stably carry a recombinant expression vector. Examples of suitable hosts include, but are not limited to, bacteria, yeast, fungi, insect cells, plant cells and mammalian cells. Generally, cells of the

expression system are the progeny of a single ancestral transformed cell.

The cloned modified receptor DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant modified receptor. Techniques for such manipulations are fully described in Sambrook, J., et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells, fungal cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungal cells or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant modified receptor in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant modified receptor expression, include but are not limited to, pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC

37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and λZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant modified receptor in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant modified receptor expression include, but are not limited to pET11a (Novagen), lambda gt11 (Invitrogen), pcDNAII (Invitrogen), pKK223-3 (Pharmacia).

A variety of fungal cell expression vectors may be used to express recombinant modified receptor in fungal cells. Commercially available fungal cell expression vectors which may be suitable for recombinant modified receptor expression include but are not limited to pYES2 (Invitrogen), *Pichia* expression vector (Invitrogen).

A variety of insect cell expression vectors may be used to express recombinant receptor in insect cells. Commercially available insect cell expression vectors which may be suitable for recombinant expression of modified receptor include but are not limited to pBlue Bac III (Invitrogen).

An expression vector containing DNA encoding modified receptor may be used for expression of modified receptor in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to Drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL

92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C1271 (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, lipofection, protoplast fusion, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce modified receptor protein. Identification of modified receptor expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-modified receptor antibodies.

Expression of modified receptor DNA may also be performed using *in vitro* produced synthetic mRNA or native mRNA. Synthetic mRNA or mRNA isolated from modified receptor producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being preferred.

The term "substantial homology", when referring to polypeptides, indicates that the polypeptide or protein in question exhibits at least about 30% homology with the naturally occurring protein in question, usually at least about 65% homology.

The modified receptors may be expressed in an appropriate host cell and used to discover compounds that affect the modified receptor. Preferably, the modified receptors are expressed in a mammalian cell line, including but not limited to, COS-7, CHO or L cells, or an insect cell line, including but not limited to Sf9 and Sf21, and may be used to discover ligands that bind to the receptor and alter or stimulate its function. The modified receptors may also be produced in bacterial, fungal or yeast expression systems.

The expression of the modified receptor may be detected by use of a radiolabeled ligand specific for the receptor. For the β_2

adrenergic receptor used herein to exemplify the invention, such a ligand may be ^{125}I-iodocyanopindolol (^{125}I-CYP).

The specificity of binding of compounds showing affinity for the modified receptors is shown by measuring the affinity of the compounds for cells transfected with the cloned modified receptor or for membranes from these cells. Expression of the cloned modified receptor and screening for compounds that inhibit the binding of radiolabeled ligand to these cells provides a rational way for selection of compounds with high affinity for the receptor. These compounds may be agonists or antagonists of the receptor. Because the modified receptor does not couple well to G proteins, the agonist activity of these compounds is best assessed by using the wild-type receptor, either natively expressed in tissues or cloned and exogenously expressed.

Once the modified receptor is cloned and expressed in a mammalian cell line, such as COS-7 cells or CHO cells, the recombinant modified receptor is in a well-characterized environment. The membranes from the recombinant cells expressing the modified receptor are then isolated according to methods known in the art. The isolated membranes may be used in a variety of membrane-based receptor binding assays. Because the modified receptor has a high affinity for agonists, ligands (either agonists or antagonists) may be identified by standard radioligand binding assays. These assays will measure the intrinsic affinity of the ligand for the receptor.

The present invention provides methods of generating modified G-protein coupled receptors. Such methods generally comprise the deletion of at least one nucleotide from the third intracellular domain of the receptor. Additional methods include, but are not limited to, enzymatic or chemical removal of amino acids from the third intracellular domain of the receptor. One method of generating modified G-protein receptors comprises:

(a) isolating DNA encoding a G-protein coupled receptor;

(b) altering the DNA of step (a) by deleting at least one nucleotide from DNA encoding the third intracellular domain of the G-protein coupled receptor;

(c) isolating the altered DNA;

(d) expressing the altered DNA; and

(e) recovering the modified G-protein coupled receptor.

The third intracellular domain of a G-protein coupled receptor is located between the fifth and sixth hydrophobic transmembrane domains of the receptor (Figure 2).

The present invention provides methods of identifying compounds that bind to modified G-protein coupled receptors. Methods of identifying compounds are exemplified by an assay, comprising:

a) cloning the G-protein coupled receptor;

b) altering the DNA sequence encoding the third intracellular domain of the cloned G-protein coupled receptor;

c) splicing the altered receptor into an expression vector to produce a construct such that the altered receptor is operably linked to transcription and translation signals sufficient to induce expression of the receptor upon introduction of the construct into a prokaryotic or eukaryotic cell;

d) introducing the construct into a prokaryotic or eukaryotic cell which does not express the altered receptor in the absence of the introduced construct; and

e) incubating cells or membranes isolated from cells produced in step c with a quantifiable compound known to bind to the receptors, and subsequently adding test compounds at a range of concentrations so as to compete the quantifiable compound from the receptor, such that an IC50 for the test compound is obtained as the concentration of test compound at which 50% of the quantifiable compound becomes displaced from the receptor.

The present invention is also directed to methods for screening for compounds which modulate the expression of DNA or RNA encoding modified receptors or which modulate the function of modified receptor protein. Compounds which modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding

modified receptor, or the function of modified receptor protein. Compounds that modulate the expression of DNA or RNA encoding modified receptor or the function of modified receptor protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample.

Kits containing modified receptor DNA, antibodies to modified receptor, or modified receptor protein may be prepared. Such kits are used to detect DNA which hybridizes to modified receptor DNA or to detect the presence of modified receptor protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic, taxonomic or epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of modified receptor DNA, modified receptor RNA or modified receptor protein. The recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of modified receptor. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant modified receptor protein or anti-modified receptor antibodies suitable for detecting modified receptor. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Pharmaceutically useful compositions comprising modulators of modified receptor activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable

composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they

may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

The modified G-protein coupled receptors of the present invention are exemplified herein by the hamster beta-2 (β_2) adrenergic receptor, the human β3 receptor and the human 5HT-1Dβ receptor.

Deletion mutagenesis of the β2-adrenergic receptor has shown that none of the hydrophobic clusters of amino acids (the putative transmembrane helices) could be deleted without substantial loss of binding. In contrast, most of the connecting loops could be deleted without affecting the ligand binding properties of the receptor. This indicates that these hydrophilic loops are not required for ligand binding to the receptor, suggesting that the ligand binding pocket is located predominantly within the transmembrane domain of the protein (Strader, et al *FASEB J* .3: 182-183 (1989)). Deletions in the connecting loops that were large enough to encompass the entire loop led to steric problems, resulting in incorrect processing of the protein (Dixon, et al. *EMBO J.* 6: 3269-3275 (1987)). Certain connecting loop deletion mutations, however, led to loss of functional activation of adenylyl cyclase by the receptor. For example, deletion of the carboxy terminal region of the third intracellular loop attenuated the ability of the receptor to activate adenylyl cyclase, and deletion of the amino terminal portion of this loop abolished adenylyl cyclase activation (Strader, et al *J. Biol. Chem.* 262: 16439-16443 (1987)). Moreover, the agonist binding isotherms for these modified receptors displayed a single affinity site, suggesting altered G protein interactions. Since these modified receptors also retain their functional activation of Na^+-H^+ exchange, which is mediated through a different G protein (Barber, et al. *Mol. Pharm.* 41: 1056-1060 (1992)), the deletions appear not to result in gross structural perturbations of the receptor, suggesting that the changes seen in adenylyl cyclase activation are due to alteration of a specific G protein interaction. Subsequent amino acid replacements in the third intracellular loop confirmed the role of this region in G protein interaction (Cheung, et al. *Mol. Pharm.* 41: 1061-1065 (1992)).

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of the examples.

EXAMPLE 1

Deletion of 6-12 amino acids at the N-terminal portion of the third intracellular loop of the hamster β2 adrenergic receptor

Modified receptor D(222-229)β2AR was described in Strader et al. (*J. Biol. Chem.* 262:16349, 1987). A modified cDNA encoding the hamster β2AR in which residues 222-229 (Val-Phe-Gln-Val-Ala-Lys-Arg-Gln) are deleted was constructed by standard oligonucleotide-directed mutagenesis procedures.

The modified receptor is designed so as to disrupt the proximal portion of the third intracellular loop, without affecting the adjacent fifth transmembrane helix. Thus, the charged amino acid (Arg221) that delineates the bottom of helix 5 is left intact in the D(222-229) modified receptor, while the following eight amino acids are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in these regions, beginning immediately after the charged residue at the end of transmembrane helix 5.

EXAMPLE 2

Deletion of amino acids at the C-terminal portion of the third intracellular loop of the hamster β2 adrenergic receptor

Modified receptor D(258-270)β2AR was described in Strader et al. (*J. Biol. Chem.* 262:16349, 1987). A modified cDNA encoding the hamster β2AR in which residues 258-270 (Leu-Arg-Arg-Ser-Ser-Lys-Phe-Cys-Leu-Lys-Glu-His-Lys) were deleted was constructed by standard oligonucleotide-directed mutagenesis procedures.

The modified receptor is designed so as to disrupt the distal portion of the third intracellular loop, without affecting the adjacent sixth transmembrane helix. Thus, the charged amino acid (Lys273) that delineates the bottom of helix 6 is left intact in the D(258-270) modified receptor, while the nearby proximal residues 258-270 are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in these regions, ending 1-3 residues before the charged residue at the beginning of helix 6.

EXAMPLE 3

Expression and characterization of the altered β2 adrenergic receptor.

COS-7 cells are transfected with the modified receptor cDNA subcloned into a eukaryotic expression vector such as the eukaryotic expression vector pcDNA I/neo (Invitrogen). Cells are harvested after incubation for about 60-72 h. Membranes containing the expressed receptor protein are prepared as described (C. D. Strader et al., *Proc. Natl. Acad. Sci. U.S.A.* 84, 4384-4388 (1987).

Binding reactions are performed in a final volume of 250 µl of TME buffer (75 mM Tris; 12.5 mM $MgCl_2$; 1.5 mM EDTA, pH 7.5) as described (Strader, et al *J. Biol. Chem.* 262: 16439 (1987)). Adenylyl cyclase activity is measured as described (Strader, et al *J. Biol. Chem.* 262: 16439 (1987)), with cAMP determined by the method of Salomon (*Anal. Biochem.* 58: 541-548 (1974)).

Membranes prepared from the COS-7 cells transfected with a vector containing either the wild type or the modified receptor cDNA specifically bind the β receptor antagonist ^{125}I-CYP. However, the modified receptor is characterized by an absence of coupling to Gs, an inability to mediate the activation of adenylyl cyclase, and an increased affinity for agonists.

As shown in Table 1, the modified D(222-229)β_2AR, when expressed in L cells, does not stimulate adenylyl cyclase activation in response to the agonist isoproterenol. In contrast, when the wild type receptor is expressed in the same cell line, adenylyl cyclase activity is stimulated by 3.2 fold, with an EC_{50} of 15 nM. The modified receptor retains its ability to stimulate Na^+-H^+ exchange, indicating that some level of coupling to a G-protein other than G_S is retained (Barber et al. Mol. Pharm. 41, 1056, 1992). Similarly, D(258-270)βAR shows impaired cAMP stimulation compared to the wild type receptor, with only a small (1.3 fold) stimulation over basal levels.

These modified receptors have increased affinity for agonists when compared to the wild type receptor. This is shown in Table 1, where the modified D(222-229) receptor binds the agonist

isoproterenol with a single high affinity of 6 nM. The high affinity of the agonist for the modified receptor is not affected by agents that uncouple the receptor from the G protein; such agents include the nonhydrolyzable GTP analog GppNHp, sodium fluoride, and the detergent digitonin. In contrast, the wild type receptor binds isoproterenol with two affinity states: a high affinity state (K_d = 3 nM) indicative of binding to the receptor-G protein complex, and a low affinity state (K_d = 200 nM) reflecting binding to the uncoupled receptor alone (Table 1). In the presence of agents that interfere with G protein coupling (GppNHp is such an agent shown in Table 1), the agonist binds to the wild type receptor with a single low affinity state (K_d = 200 nM).

The data in Table 1 demonstrate that when the receptor is not optimally coupled to the G protein, a binding assay using the modified receptor will detect agonists with more sensitivity than will the identical binding assay using the wild type receptor. Similarly, D(258-270)ßAR binds to the agonist isoproterenol with a single high affinity of 8 nM, which is not significantly affected by the addition of Gpp(NH)p.

EXAMPLE 4

Screening Assay using D(222-229) βAR or D(258-270)βAR

Transfected cells expressing recombinant modified receptor may be used to identify compounds that bind to the receptor with high affinity. This may be accomplished in a variety of ways, such as by incubating the test compound in a final volume of 0.25 ml of TME buffer with membranes containing 5-7 pM of the modified β_2AR and 35 pM ^{125}I-CYP for 1 hour at 25°. The reaction is stopped by filtration over GF/C glass fiber filters, washing with 3 x 5 ml of cold TME buffer, and counting the filters in a gamma counter to measure bound radioactivity. This assay will detect a compound that has a high intrinsic affinity for the receptor. Such compounds may be either agonists or antagonists.

EXAMPLE 5

Construction of Modified D(227-234) Beta-3 Adrenergic Receptor

Modified receptor D(227-234) β3AR was constructed by digesting the wild-type human β3 receptor cDNA (Granneman, et al. *Mol. Pharm.* 42: 964-970 (1992))) with AccI and PvuII, followed by re-ligation with a linker adaptor. The sequence of the linker adaptor is:

5'CTACGCGCGG3'/3'TGCGCGCC5' (SEQ ID NO:1).

The modified DNA sequence encodes a β3AR lacking 8 amino acid residues (VFVVATRQ) at the N-terminal portion of the third intracellular loop. The nucleotide sequence of the modified receptor was confirmed by DNA sequencing. As was the case for the modified β2 receptors, this modified β3 receptor is designed so as to disrupt the proximal portion of the third intracellular loop, without affecting the adjacent fifth transmembrane helix. Thus, the charged amino acid (Arg226) that delineates the bottom of helix 5 is left intact in the D(227-234) modified receptor, while the eight amino acids which follow it are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, beginning immediately after the charged residue at the bottom of transmembrane helix 5.

EXAMPLE 6

Construction of Modified D(277-289) Beta-3 Adrenergic Receptor

Modified D(277-289), lacking 13 residues at the C-terminal portion of the third intracellular loop, was prepared by standard PCR-based mutagenesis procedures. The nucleotide sequences of the modified receptors were confirmed by DNA sequencing. As was the case for the modified β2 receptors, this modified β3 receptor is designed so as to disrupt the distal portion of the third intracellular loop, without affecting the adjacent sixth transmembrane helix. Thus, the polar amino acids (C292,T293) that define the bottom of helix 6 are left intact, while the nearby proximal residues 277-289 are deleted. The

size of the deletion in the present invention may vary from six to 13 amino acids in this region, ending immediately before the polar residues at the bottom of helix 6.

EXAMPLE 7

Expression and characterization of the modified β3AR

The modified receptor was subcloned into the expression vector pRC/CMV (Invitrogen, San Diego, CA) and expressed in mouse L cells by DEAE-Dextran transfection. 72 hours after transfection, cells were harvested for radioligand binding or adenylyl cyclase assays.

For binding assays, the membranes were prepared by harvesting the cells in ice-cold lysis buffer (5 mg Tris, pH 7.4; 2 mM EDTA), followed by 15 min centrifugation at 38,000 x g. The membrane pellet was then resuspended in TME buffer. Equilibrium binding to the wild type or modified β3AR was performed in a final volume of 0.25 ml containing membranes, 240 pM ^{125}I-CYP, and serial dilution of the competing ligands. Binding reactions were incubated for 90 min at 23°C, and terminated by rapid filtration over GF/C filters pre-soaked in 0.1% polyethyeneamine. The radioactivity was quantified with a Packard gamma counter.

For adenylyl cyclase activity, cells are harvested in PBS with 5 mM EDTA, pelleted and, then resuspended in ACC buffer (75 mM Tris, pH 7.4; 250 mM sucrose; 12.5 mM $MgCl_2$; 1.5 mM EDTA; 1 μM ascorbic acid; 0.6 mM 3-isobutyl-1-1 - methylxanthine). The cells are incubated with various concentrations of test compound (usually agonist compound) for 45 min at room temperature, and the reaction terminated by boiling for 3 min. The concentration of cAMP in the lysate was determined via protein kinase A (PKA) binding assay (Barton, A.C., Black, L.E., Sibley, D.R., *Mol. Pharmacy.* 39:650-658, 1991) or an automated cAMP IRA assay (At Instruments, MD). For the PKA binding assay, the lysate was incubated with 3.6 nM ^{3}H-cAMP and 5 μg of PKA in a final volume of 185 μl for 2 to 24 hours at 4° C, followed by rapid filtration over GF/C filters with cold washing buffer (20 mM potassium phosphate, pH 6.0). The radioactivity on the filter

was then quantified on a beta counter. The final concentration of cAMP was determined according to the standard curve of cAMP. The data for both binding and cyclase assays were analyzed by using graphed software (San Diego, CA).

Figure 3 shows that, when stimulated with the beta agonist isoproterenol, there is a four-fold increase in the production of cAMP in L cells transfected with the wild type human β3AR, with a EC_{50} of $2.7 \pm 0.5 \times 10^{-8}$ M (n=4). By contrast, the β3AR-mediated production of cAMP is essentially abolished in cells transfected with modified receptor D(227-234)β3AR and strongly attenuated in cells expressing the D(277-289) modified receptor.

Radioligand binding with ^{125}I-CYP indicates that the wild type β3AR displays two affinity sites for isoproterenol binding: a high affinity site (28%, IC_{50}=5 x 10^{-8} M), and a low affinity site (72%, IC_{50}=2.6 x 10^{-6} M). Deletion of residues 227-234 or residues 277-289 from the β3AR results in a single high affinity binding state (Table 2 and Figure 4). No increase in binding affinity is observed for the βAR antagonist propranolol for either modified receptor (Figure 4).

These modified β3 receptors can therefore be used in a screening assay to detect compounds that bind with high affinity to the β3 adrenergic receptor, regardless of whether these compounds are agonists or antagonists.

EXAMPLE 8

Construction of Modified D(231-238)5HT-1Dβ Receptor

Modified receptor D(231-238)5HT-1Dβ receptor was constructed from the wild-type human 5HT-1Dβ receptor cDNA (Jin, et al J. Biol. Chem. 267: 5735 (1992)) by standard mutagenesis techniques. The modified 5HT-1Dβ receptor lacks 8 amino acid residues (IYVEARSR) at the N-terminal portion of the third intracellular loop. The nucleotide sequences of the modified receptors were confirmed by DNA sequencing. As was the case for the modified β2 and β3 receptors, this modified 5HT-1Dβ receptor is designed so as to disrupt the proximal portion of the third intracellular loop without affecting the

adjacent fifth transmembrane helix. Thus, the charged amino acid (Arg230) that delineates the bottom of helix 5 is left intact in the modified receptor, while the following eight amino acids are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, beginning immediately after the charged residue at the end of transmembrane helix 5.

EXAMPLE 9

Expression and Characterization of Modified D(231-238)5HT-1Dβ Receptor

The modified receptor was subcloned into a mammalian expression vector and expressed in CHO cells using standard transfection methods. Stable cell lines were selected by G-418 resistance and used for radioligand binding or adenylyl cyclase assays.

For binding assays, the membranes were prepared by harvesting the cells in ice-cold lysis buffer (5 mg Tris, pH 7.4; 2 mM EDTA), followed by 15 min centrifugation at 38,000 x g. The membrane pellet was then resuspended in buffer A. Equilibrium binding to the wild type or modified 5HT-1Dβ was performed in a mixture containing membranes, 5 nM ^{3}H 5-hydroxytryptamine, and serial dilutions of the competing ligands. Binding reactions were incubated for x min at 23°C, and terminated by rapid filtration over GF/C filters. The bound radioactivity was quantified with a gamma counter.

Adenylyl cyclase activity was measured essentially as described by McAllister et al. (McAllister, G., Charlesworth, A., Snodin, C., Beer, M. S., Noble, A. J., Middlemiss, D. N., Iversen, L. L., and Whiting, P., 1992, PNAS 89:5517-5521), with the addition of forskolin. Inhibition of the forskolin-stimulated response by receptor agonists, including 5-hydroxytryptamine (serotonin), was determined.

Figure 5 shows that, when stimulated with the agonist serotonin, there is a 50% inhibition in the forskolin-stimulated production of cAMP in cells expressing with the wild type human 5HT-1Dβ receptor, with a EC_{50} of 30 nM. By contrast, the agonist-

mediated inhibition of cAMP production is essentially abolished in cells transfected with modified receptor D(231-239)5HT-1Dβ.

Radioligand binding studies at the wild type 5-HT1Dβ receptor indicate that when the guanine nucleotide analogue, GppNHp (guanylylimidodiphosphate) is present (100 mM), agonist binding (2 nM ^{3}H-5-HT) is reduced by approximately 50-60% (Table 3). This is thought to be a result of the guanine nucleotide converting the receptor to the low affinity state. However, in three independent clones expressing the modified receptor, D(231-239)5-HT1Dβ (clones 1, 21 and 65), no significant inhibition of agonist binding is observed, suggesting that the modified receptor is permanently in the high affinity state.

This modified 5HT-1Dβ receptor can therefore be used in a screening assay to detect compounds that bind with high affinity to the 5HT-1Dβ receptor, regardless of whether these compounds are agonists or antagonists.

EXAMPLE 10

Cloning and Expression of Modified Receptor cDNA into Bacterial Expression Vectors

Recombinant modified receptor is produced in a bacterial expression system such as E. coli. The modified receptor expression cassette is transferred into an E. coli expression vector; expression vectors include but are not limited to, the pET series (Novagen). The pET vectors place modified receptor expression under control of the tightly regulated bacteriophage T7 promoter. Following transfer of this construct into an E. coli host which contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of modified receptor is induced by addition of an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed modified receptor are determined by the assays described herein.

EXAMPLE 11

Cloning and Expression of Modified Receptor cDNA into a Vector for Expression in Insect Cells

Baculovirus vectors derived from the genome of the AcNPV virus are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculovirus expressing modified receptor cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the modified receptor cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P.A., *Nuc. Acid. Res.* 18, 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of ß-galactosidase expression (Summers, M. D. and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, modified receptor expression is measured.

Authentic modified receptor is found in association with the infected cells. Active modified receptor is extracted from infected cells by hypotonic or detergent lysis.

Alternatively, the modified receptor is expressed in the *Drosophila* Schneider 2 cell line by cotransfection of the Schneider 2 cells with a vector containing the modified receptor DNA downstream and under control of an inducible metallothionin promoter, and a vector encoding the G418 resistant neomycin gene. Following growth in the presence of G418, resistant cells are obtained and induced to express modified receptor by the addition of $CuSO_4$. Identification of modulators of the modified receptor is accomplished by assays using either whole cells or membrane preparations.

EXAMPLE 12

Cloning of Modified Receptor cDNA into a yeast expression vector

Recombinant modified receptor is produced in the yeast S. cerevisiae following the insertion of the modified receptor cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of heterologous proteins. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the modified receptor cistron [Rinas, U. et al., *Biotechnology* 8, 543-545 (1990); Horowitz B. et al., *J. Biol. Chem.* 265, 4189-4192 (1989)]. For extracellular expression, the modified receptor cistron is ligated into yeast expression vectors which fuse a secretion signal. The levels of expressed modified receptor are determined by the assays described herein.

EXAMPLE 13

Purification of Recombinant Modified Receptor

Recombinantly produced modified receptor may be purified by a variety of procedures, including but not limited to antibody affinity chromatography.

Modified receptor antibody affinity columns are made by adding the anti-modified receptor antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1 M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents, and the cell culture supernatants or cell extracts containing solubilized modified receptor or modified receptor subunits are slowly passed through the column. The column is then washed with phosphate-buffered saline (PBS)

supplemented with detergents until the optical density (A_{280}) falls to background; then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) supplemented with detergents. The purified modified receptor protein is then dialyzed against PBS.

EXAMPLE 14

Cloning and Expression of Modified Receptor in Mammalian Cell System

A modified receptor is cloned into a mammalian expression vector. The mammalian expression vector is used to transform a mammalian cell line to produce a recombinant mammalian cell line. The recombinant mammalian cell line is cultivated under conditions that permit expression of the modified receptor. The recombinant mammalian cell line or membranes isolated from the recombinant mammalian cell line are used in assays to identify compounds that bind to the modified receptor.

EXAMPLE 15

Screening Assay

Recombinant cells containing DNA encoding a modified receptor, membranes derived from the recombinant cells, or recombinant modified receptor preparations derived from the cells or membranes may be used to identify compounds that modulate modified G-protein coupled receptor activity. Modulation of such activity may occur at the level of DNA, RNA, protein or combinations thereof. One method of identifying compounds that modulate modified G-protein coupled receptor, comprises:

(a) mixing a test compound with a solution containing modified G-protein coupled receptor to form a mixture;

(b) measuring modified G-protein coupled receptor activity in the mixture; and

(c) comparing the modified G-protein coupled receptor activity of the mixture to a standard.

WHAT IS CLAIMED IS:

1. Isolated DNA encoding a modified receptor, the modified receptor being derived from a G-protein coupled receptor having seven transmembrane domains and the modified receptor having deletions in the third intracellular domain, or a functional derivative thereof.

2. The DNA of Claim 1 wherein the modified receptor is a modified β3-adrenergic receptor.

3. Isolated RNA encoded by the isolated DNA of Claim 1 or its complementary sequence.

4. Isolated RNA encoded by the isolated DNA of Claim 2 or its complementary sequence.

5. An expression vector containing the isolated DNA of Claim 1.

6. A recombinant host cell containing the expression vector of Claim 5.

7. A process for the production of a modified G-protein coupled receptor, comprising:

a) transforming a host cell with the isolated DNA of Claim 1 to produce a recombinant host cell;

b) culturing the recombinant host cell under conditions which allow the production of modified G-protein coupled receptor; and

c) recovering the modified G-protein coupled receptor.

8. The modified G-protein coupled receptor produced by the process of Claim 7.

9. The process of Claim 7 wherein the modified G-protein coupled receptor is a modified beta-3 adrenergic receptor.

10. An isolated and purified modified G-protein coupled receptor, the receptor having seven transmembrane domains and having amino acids deleted from the third transmembrane domain, or a functional derivative thereof.

11. The purified modified G-protein coupled receptor of Claim 10 which is a modified beta-3 adrenergic receptor.

12. A method of identifying compounds that modulate modified G-protein coupled receptor activity, comprising:

(a) mixing a test compound with a solution containing modified G-protein coupled receptor to form a mixture;

(b) measuring modified G-protein coupled receptor activity in the mixture; and

(c) comparing the modified G-protein coupled receptor activity of the mixture to a standard.

13. Compounds identified by the method of Claim 12.

14. Pharmaceutical compositions comprising the compound of Claim 13.

15. A method for identifying compounds which specifically bind to a modified G-protein coupled receptor, comprising:

(a) cloning a G-protein coupled receptor;

(b) altering the DNA sequence encoding the third intracellular domain of the cloned G-protein coupled receptor;

(c) splicing the altered receptor into an expression vector to form a construct;

(d) introducing the construct into a cell which does not express the altered receptor in the absence of the introduced construct;

(e) incubating cells or membranes isolated from cells produced in step c with a quantifiable compound known to bind to the receptor; and

(f) adding test compounds so as to compete the quantifiable compound from the receptor.

16. Compounds identified by the method of Claim 15.

17. A method of making a modified G-protein coupled receptor, comprising:

(a) isolating DNA encoding a G-protein coupled receptor;

(b) altering the DNA of step (a) by deleting at least one nucleotide from DNA encoding the third intracellular domain of the G-protein coupled receptor;

(c) isolating the altered DNA;

(d) expressing the altered DNA; and

(e) recovering the modified G-protein coupled receptor.

18. The modified G-coupled protein receptors of Claim 17.

19. The method of Claim 17 wherein between six and thirteen nucleotides are deleted from DNA encoding the third intracellular domain of the G-protein coupled receptor.

20. The isolated DNA of Claim 1 wherein the modified G-protein coupled receptor is selected from the group consisting of:

(a) D(277-289) beta-3 adrenergic receptor; and
(b) D(227-234) beta-3 adrenergic receptor.

21. The isolated and purified receptor of Claim 10 wherein the modified beta adrenergic receptor is selected from the group consisting of D(277-289) beta-3 adrenergic receptor and D(227-234) beta-3 adrenergic receptor.

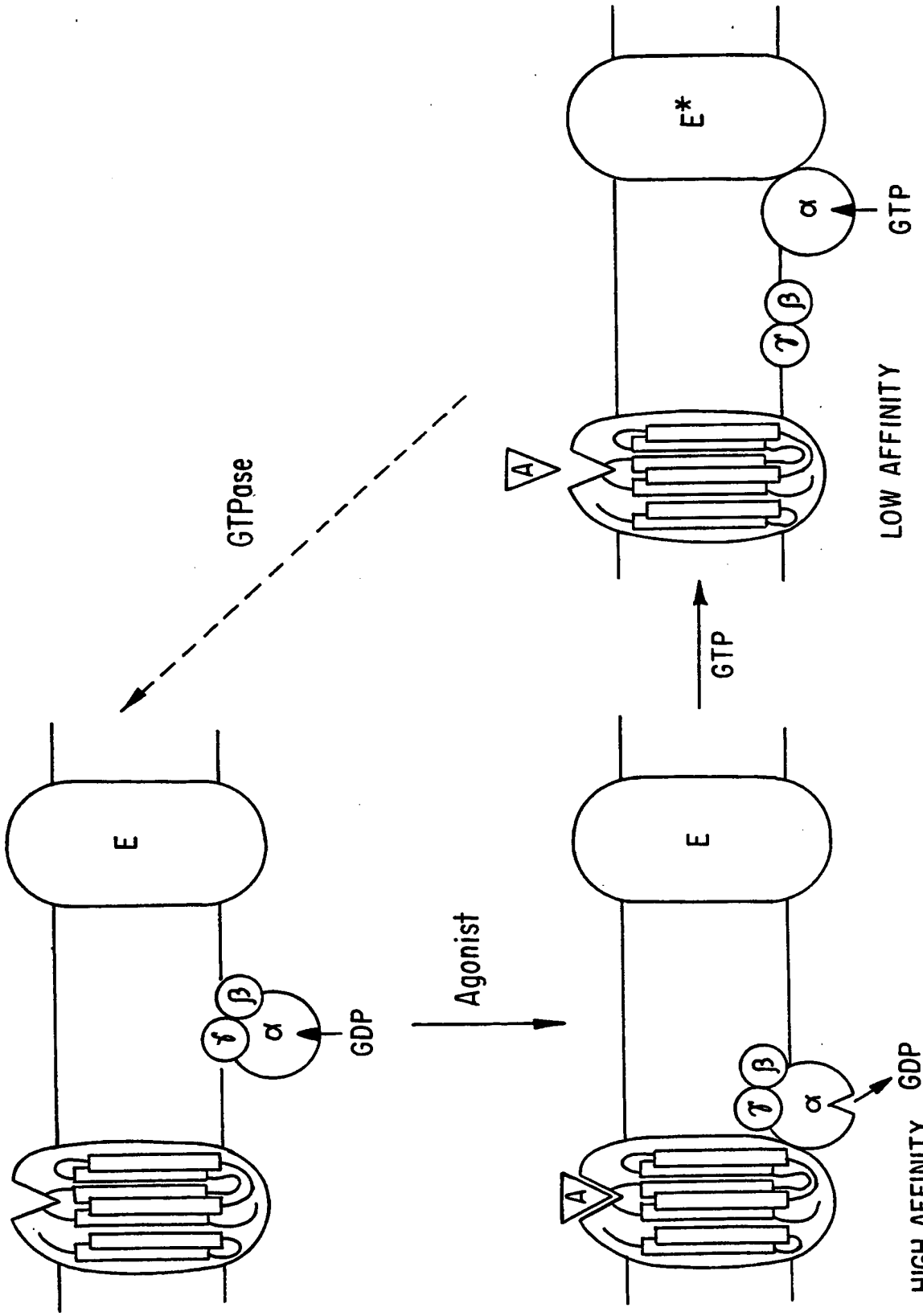
E*
α
GTP
γ
β
A
LOW AFFINITY
GTPase
GTP
E
E
Agonist
γ
β
α
GDP
A
γ
β
α
GDP
HIGH AFFINITY

FIG. 1

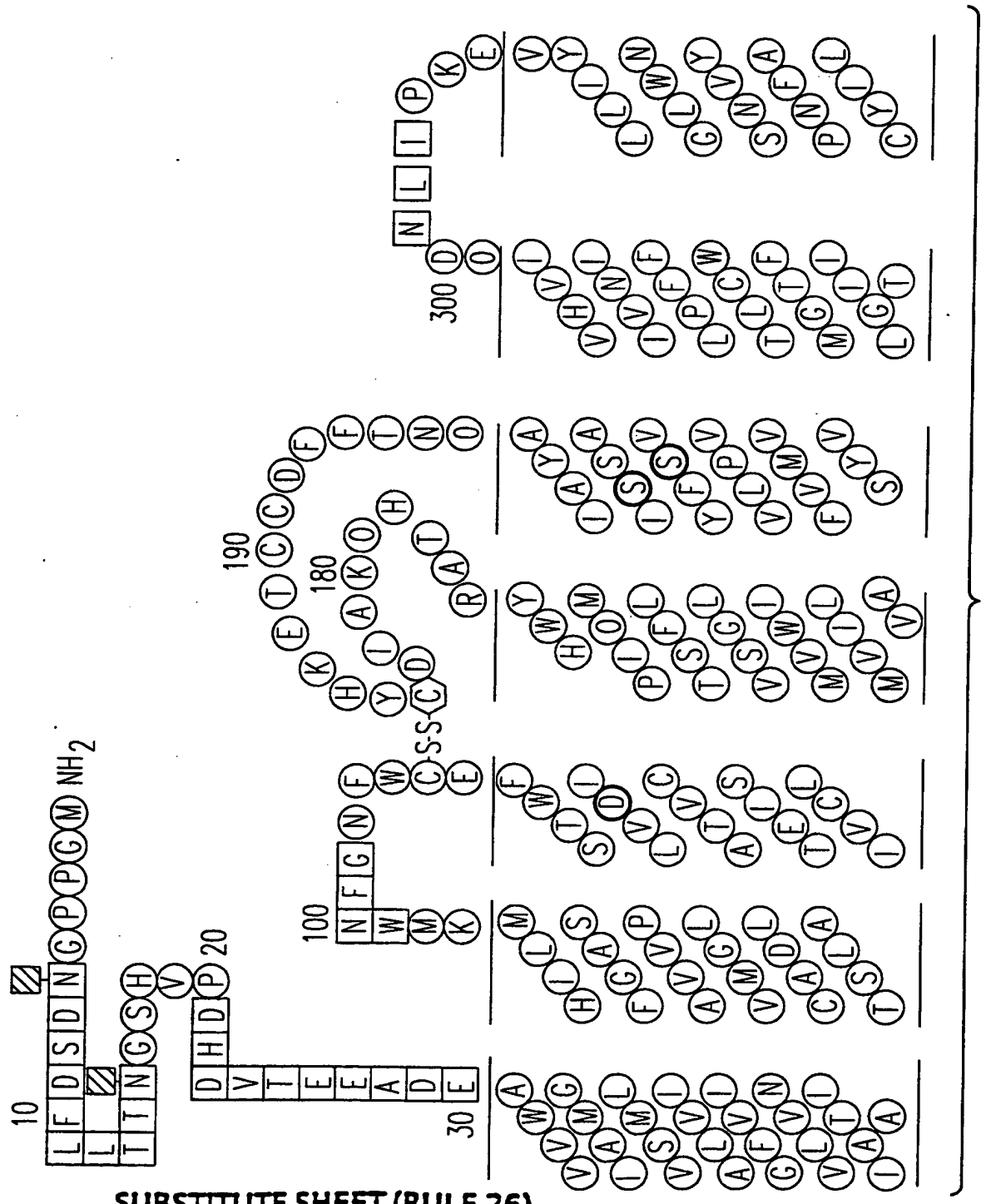

FIG.2A

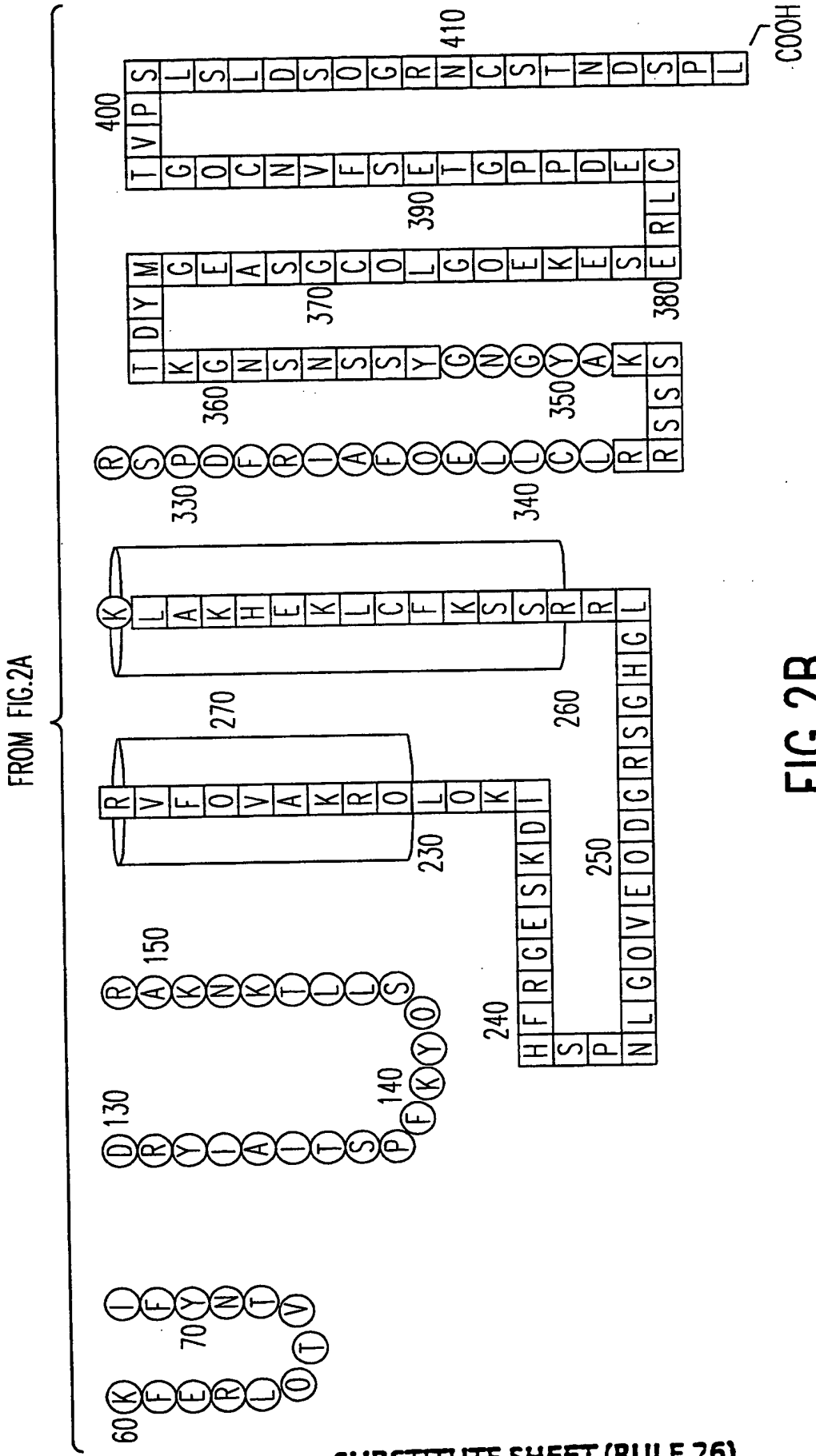
FROM FIG.2A
COOH
410
400
390
380
370
360
350
340
330
270
260
250
240
230
150
140
130
70
60

FIG.2B

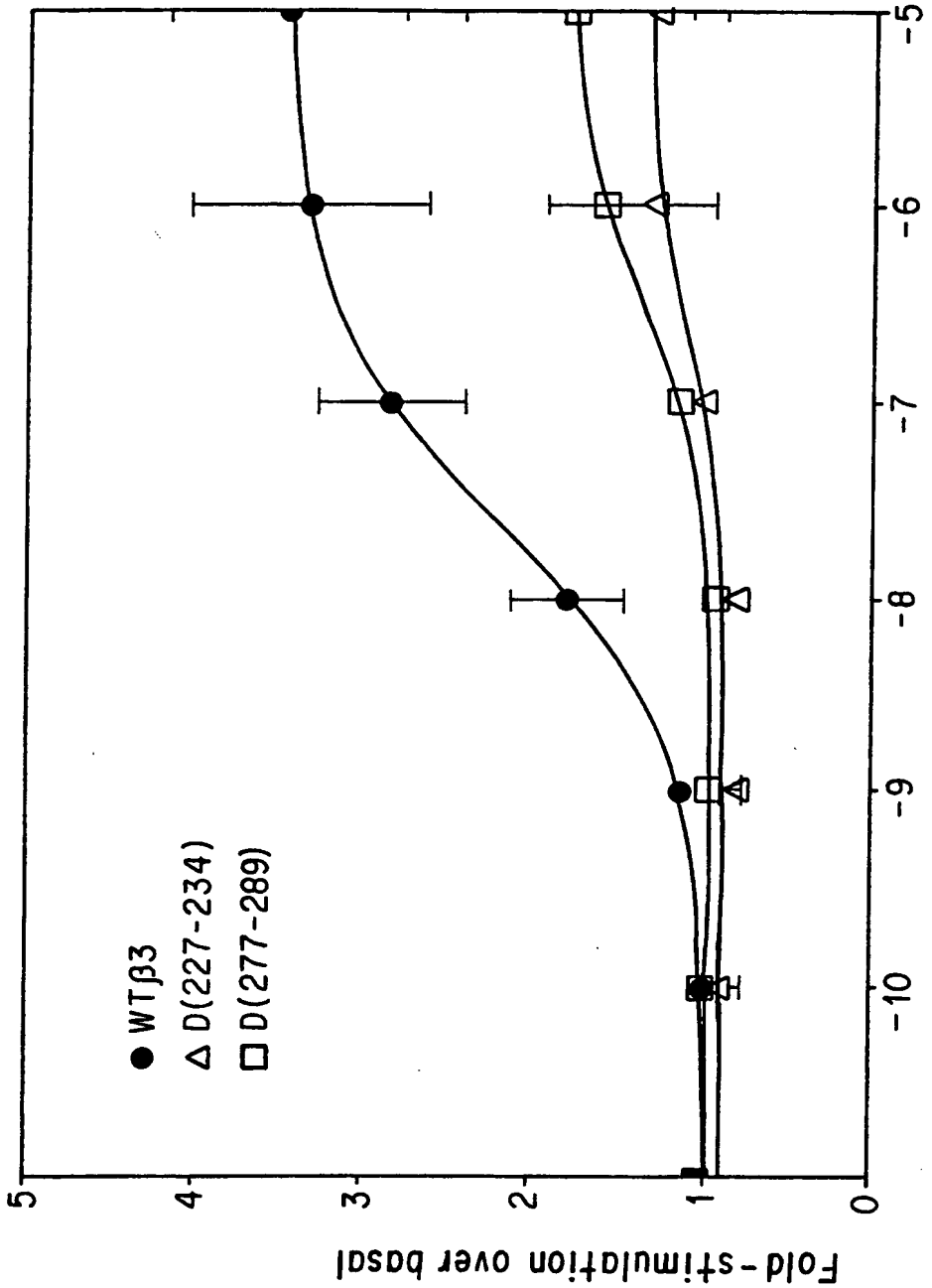
Fold-stimulation over basal
0
1
2
3
4
5
-5
-6
-7
-8
-9
-10
Log [isoproterenol], M
● WTβ3
△ D(227-234)
□ D(277-289)

FIG. 3

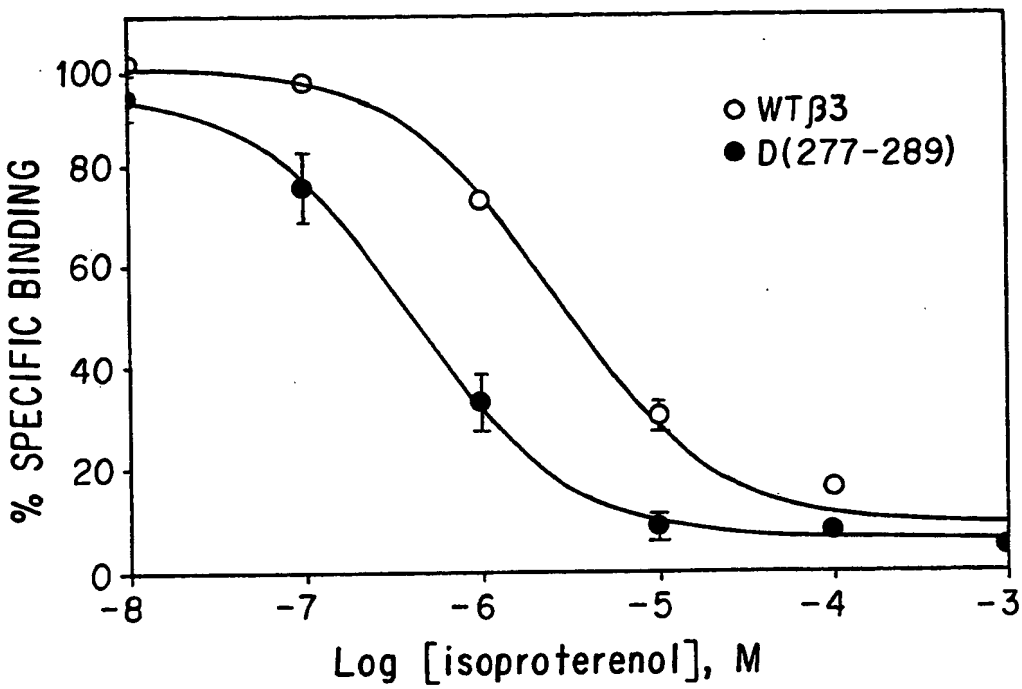
100
80
60
40
20
0
% SPECIFIC BINDING
WTβ3
D(277-289)
-8
-7
-6
-5
-4
-3
Log [isoproterenol], M

FIG. 4A

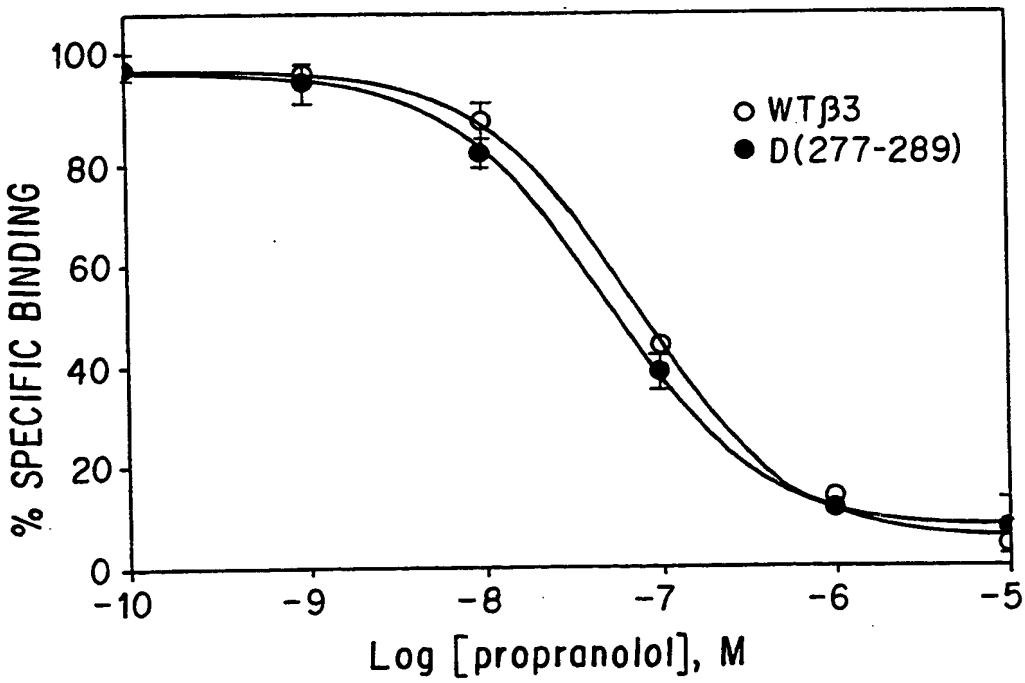
100
80
60
40
20
0
% SPECIFIC BINDING
WTβ3
D(277-289)
-10
-9
-8
-7
-6
-5
Log [propranolol], M

FIG. 4B

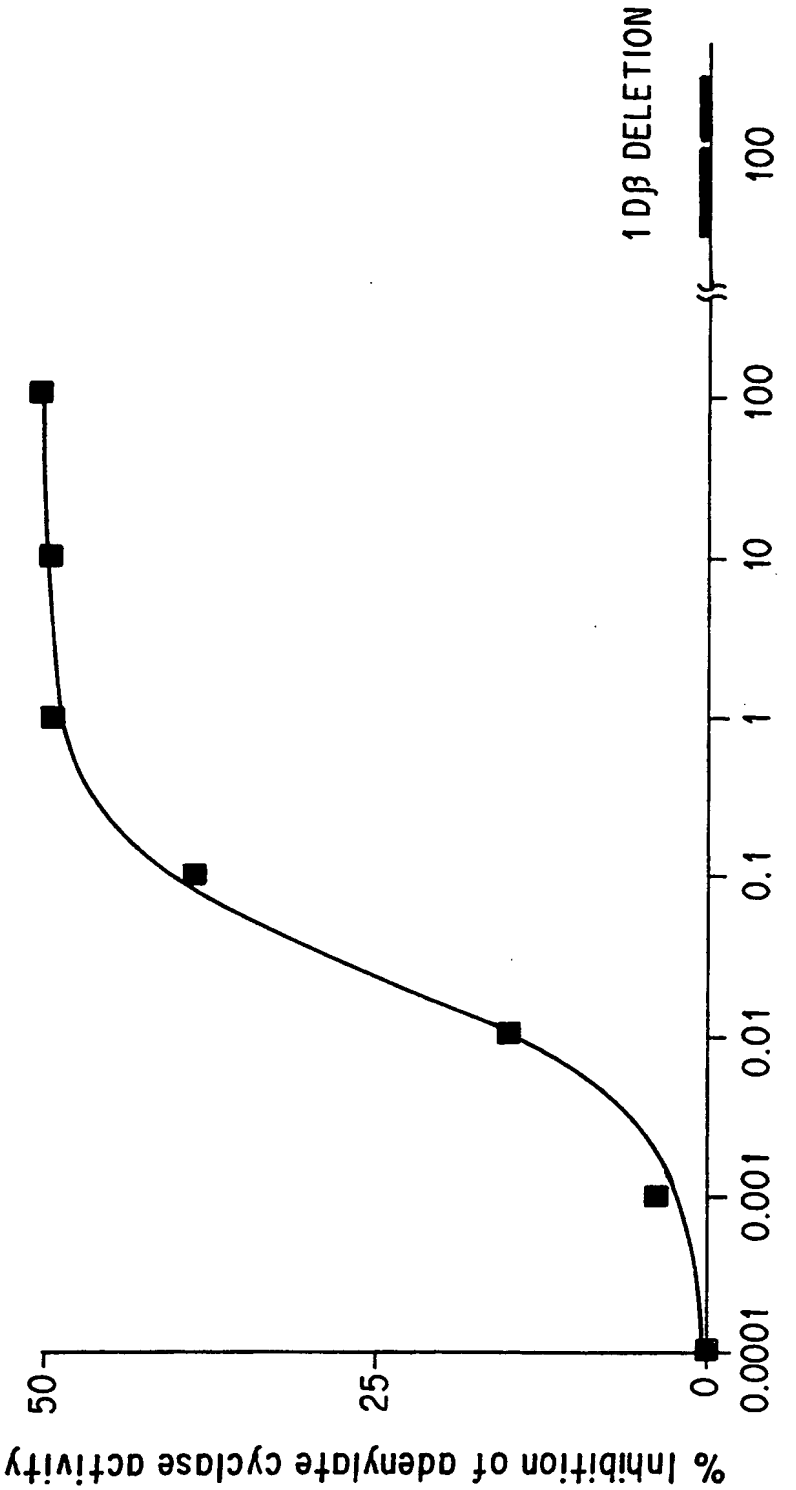
% Inhibition of adenylate cyclase activity
50
25
0
0.0001
0.001
0.01
0.1
1
10
100
[5-HT] (μM)
1 Dβ DELETION
100

FIG. 5

PROPERTIES OF MUTANT β2 RECEPTORS						
	Kd (nM)					
	– Gpp(NH)p			+Gpp(NH)p	Adenylyl cyclase activation	
Mutant	K_H	K_L	%H	Kd	Kact (nM)	fold stimulation
Wild type βAR	3	200	70	200	15	3.2
D(222-229)βAR	6	—	100	7	—	1.0
D(258-270)βAR	8	—	100	10	—	1.3

FIG. 6

	Binding affinity (M)				cAMP production	
	K_H	$\%K_H$	K_L	$\%K_L$	Max stimulation (fold over basal)	Kact (M)
WT β3AR	$5.0 \pm 2.1 \times 10^{-8}$	28 ± 4	$2.6 \pm 0.7 \times 10^{-6}$	72 ± 4	3.5 ± 0.7	$2.6 \pm 0.3 \times 10^{-8}$
D227-234	$1.8 \pm 1.0 \times 10^{-7}$	100	n.d		1.3 ± 0.4	n.d
D277-289	$2.2 \pm 0.4 \times 10^{-7}$	87 ± 13	4.4×10^{-6}*	13 ± 13	1.8 ± 0.7	n.d

n.d. not detectable.
*The low affinity binding site was detected in only one experiment.

FIG. 7

PCT/US95/06900

SUBSTITUTE SHEET (RULE 26)

Radialigand binding properties of mutant 5HT1Dβ receptors				
Receptor	Specific Binding (dpm)	Specific Binding (+GppNHp) (dpm)	% Guanine nucleotide Shift	% Adenylate Cyclase Inhibition
Wild Type	4120	1553	67	45.6
D(231-239)1Dβ Clone1	2046	1885	8	-4.9
D(231-239)1Dβ Clone21	716	710	1	-3.3
D(231-239)1Dβ Clone65	1450	1292	11	0.3

FIG. 8

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06900

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/350; 435/7.1, 7.2, 69.1, 172.1, 240.1, 252.3, 254.11, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Dialog, WPI
search terms: β3-Adrenergic Receptor, modified, variant, deletions in the third intracellular domain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular Pharmacology, Volume 37, Number 6, issued June 1990, Cheung et al, "Separation of the Structural Requirements for Agonist-Promoted Activation and Sequestration of the β-Adrenergic Receptor", pages 775-779, see pages 776-777.	1-21
Y	Science, Volume 245, issued 08 September 1989, Emorine et al, "Molecular Characterization of the Human β_3-Adrenergic Receptor", pages 1118-1121, see entire document.	1-21

[X] . Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 AUGUST 1995	Date of mailing of the international search report 15 SEP 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Sally P. Teng Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06900

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular Pharmacology, Volume 38, Number 3, issued September 1990, Johnson et al, "Identification of a Specific Domain in the β-Adrenergic Receptor Required for Phorbol Ester-Induced Inhibition of Catecholamine-Stimulated Adenylyl Cyclase", pages 289-293, see pages 292-293.	1-21
Y	Molecular Pharmacology, Volume 40, issued 1991, Granneman et al, "Molecular Cloning and Expression of the Rat β_3-Adrenergic Receptor", pages 895-899, see pages 896-898.	1-21
Y	The Embo Journal, Volume 10, Number 12, issued 1991, Nahmias et al, "Molecular Characterization of the Mouse β3-Adrenergic Receptor: Relationship with the Atypical Receptor of Adipocytes", pages 3721-3727, see pages 3722-3724.	1-21
Y	Molecular Pharmacology, Volume 44, issued September 1993, Proll et al, "β_2-Adrenergic Receptor Mutants Reveal Structural Requirements for the Desensitization Observed with Long Term Epinephrine Treatment", pages 569-574, see pages 570-574.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06900

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): C07K 14/705; C07H 21/00; C12N 5/10, 15/63, 15/70, 15/79; C12Q 1/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL : 536/23.1; 530/350; 435/7.1, 7.2, 69.1, 172.1, 240.1, 252.3, 254.11, 320.1